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Functional Dissection of the Proteins of the Endoplasmic Reticulum Associated Degradation (ERAD) Pathway

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Shivanjali Joshi

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Shivanjali Joshi

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

William J. Lennarz Ph.D. – Dissertation Advisor
Distinguished Professor,
Department of Biochemistry and Cell Biology, Stony Brook University

Deborah Brown Ph.D. – Chairperson of Defense
Professor, Department of Biochemistry and Cell Biology,
Stony Brook University

Erwin London, Ph.D.
Professor, Department of Biochemistry and Cell Biology,
Stony Brook University

Jeffery Pessin, Ph.D.
Professor and Chair, Department of Pharmacology,
Stony Brook University

Allan M. Weissman, M.D.
Laboratory Chief, Laboratory of Protein Dynamics and Signaling
Center for Cancer Research, National Cancer Institute

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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Newly synthesized glycoproteins acquire their "folded" conformation by concerted action of chaperones in the lumen of the endoplasmic reticulum (ER). Glycoproteins that fail to fold correctly are transported to the cytosol and are eventually degraded by the 26S proteasome, by a process known as ER associated degradation (ERAD). My dissertation evaluates the role of three proteins, namely peptide: N-glycanase (PNGase), gp78 and Derlin-1, in the ERAD process.

PNGase is a cytosolic deglycosylating enzyme that cleaves N-linked glycans. By developing an *in vitro* assay, I have shown that, a) PNGase deglycosylates full-length glycoproteins and not just glycopeptides as previously believed; b) glycoprotein misfolding is a prerequisite for PNGase mediated action. My findings on the subcellular localization of PNGase in HeLa cells show that a minor fraction of PNGase is tethered to the ER via Derlin-1, an ER membrane protein. This interaction with Derlin-1, which

presumably functions in glycoprotein dislocation from the ER, brings PNGase in close proximity to misfolded glycoproteins.

Gp78, also known as autocrine motility factor receptor, was originally identified as a cell-surface glycoprotein important in tumor cell motility. Subsequently gp78 was found to function as an ER associated E3 ubiquitin-ligase involved the turnover of misfolded glycoproteins. Using a combinatorial approach of biotinylation and confocal microscopy, I have shown that gp78 localizes exclusively to the ER. Also, my analysis of gp78 using the deglycosylating enzyme Endo H refutes the notion that this protein is modified by N-linked glycans, due to the lack of its mobility shift on SDS-PAGE upon Endo H treatment.

The Derlin family of ER membrane proteins, comprised of Derlin-1, 2 and 3 in humans have been recently identified as ERAD factors involved in the dislocation of misfolded glycoproteins. To date, only four substrates of the Derlins have been identified: CPY*, MHC class I heavy chains, CFTR and α-1 antitrypsin. I have identified a novel substrate of Derlin-1: CD3-δ, a component of the T cell receptor complex. My data suggests that Derlin-1 interacts with CD3-δ and Derlin-1 overexpression in HeLa cells causes its accelerated turnover. These findings suggest the importance of Derlin-1 in the pathway for CD3-δ degradation.

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Abbreviations

ATP	adenosine 5' triphosphate
CHX	cycloheximide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EM	electron microscopy
GFP	green fluorescence protein
IP	immunoprecipitation
kDa	kilo Daltons
HC	major histocompatibilty complex
MW	molecular weight
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethanesulfonyl fluoride
rpm	revolutions per minute
RNase	ribonuclease
SDS	sodium doedcyl sulfate
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
WB	Western blotting

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Chapter1. Introduction

In eukaryotes, proteins destined for the secretory pathway are often modified by glycans on the asaparagine (Asn) residues. This phenomenon constitutes N-linked glycosylation and is essential for cell viability. The glycan moiety contributes to the overall structure and function of the modified glycoprotein and also imparts steric protection against proteolytic action.

The endoplasmic reticulum (ER) is the site for biogenesis of N-linked glycoproteins. The nascent polypeptide chain synthesized by the ribosome is translocated into the ER lumen via a pore formed by the Sec61 complex (Sec61α, Sec61β, and Sec61γ in mammals and Sec61p, Sbh1p, Sss1p in yeast) [1-3]. Concomitantly, the oligosaccharyl transferase complex transfers an oligosaccharide from a dolichol precursor to the Asn residue on the N-glycosylation consensus sequence Asn-X_{aa}-Ser/Thr (X_{aa} being any amino acid except proline) [4]. Upon completion of polypeptide translocation and glycosylation, the newly synthesized glycoprotein acquires a native or folded conformation by the concerted efforts of ER resident chaperones. The native glycoproteins enter the secretory pathway or remain in the ER as stable resident proteins. Glycoproteins may be transported to other subcellular compartments such as the Golgi, the lysosome or the plasma membrane. Glycoproteins that are unable to fold correctly are recognized by a surveillance mechanism known as ER quality control or ERQC [5]. Such aberrant glycoproteins are retained in the ER

and subsequently transported to the cytosol for proteasomal destruction by the ER-associated degradation (ERAD) pathway [6].

Persistence of misfolded glycoproteins in the ER lumen and their continued production gives rise to the unfolded protein response (UPR) [7]. The UPR is a network of cell signaling pathways that activate transcription of genes involved in the synthesis, folding and ERAD of glycoproteins, primarily to allow the cell to adjust to the load of misfolded protein. The mechanisms triggering UPR and its signaling cascades are highlighted below.

The Unfolded Protein Response

As mentioned above, the UPR is activated in response to cellular overload of misfolded proteins. Other factors, such as defects in glycosylation, redox state and nutrient starvation, which perturb the cellular milieu, also induce UPR.

A central player of the UPR is the Hsp70 family chaperone, BiP, also known as Kar2p in yeast. During glycoprotein synthesis, as the nascent polypeptide chain enters the ER lumen via the Sec61 pore, binding of BiP prevents its retrograde movement in the channel and out to the cytosol [8]. This process is energetically driven and requires ATP hydrolysis. Movement of the polypeptide chain away from the channel causes BiP dissociation. Under steady state conditions BiP also associates with UPR sensors: IRE1 (inositol requiring kinase1), PERK (double stranded RNA-activated protein kinase like ER kinase) and ATF6 (activating transcription factor 6). Upon cellular stress leading to an overload of misfolded proteins, BiP dissociates from the aforementioned proteins and binds to the misfolded protein, causing the activation of three different signaling cascades [9].

IRE1 is an ER membrane that has both protein kinase and endoribonuclease (RNase) functions [10]. Homodimerization of IRE1 occurs after dissociation from BiP and results in transautophosphorylation, which in turn activates its RNase activity in the cytosol. The RNase activity of yeast IRE1 leads to the differential splicing of Hac1 (XBP1 in metazoans) mRNA and subsequent production of Hac1 transcription factor that activates UPR target genes [11]. It is estimated that in yeast approximately 400 genes are under the transcriptional regulation of IRE1/Hac1 pathway [12].

As in the case of IRE1, PERK mediated signaling also involves homodimerization and transautophosphorylation [13]. This causes the activation of its second kinase domain, which phosphorylates eIF2 (eukaryotic translational initiation factor 2), and ultimately leads to translational attenuation. A halt in the synthesis of new proteins by this mechanism reduces the misfolded protein load in the ER. PERK activity is also responsible for the altered localization of two transcription factors NRF1 and NRF2 that are transported to the nucleus, where they activate genes encoding detoxifying enzymes [14].

ATF6 leaves the ER and enters the Golgi complex upon the dissociation of BiP. Proteolytic cleavage of ATF6 in the Golgi gives rise to a cytosolic fragment of ATF6, which migrates to the nucleus and binds promoters containing ERSE (ER stress response elements), thereby initiating transcription of UPR target genes [15]. Thus, UPR signaling cascade safeguards the cell by maintaining homeostasis.

Folding and Quality Control in the ER

As mentioned earlier, a glycoprotein must adopt its native conformation in order to be functional. Hence it must undergo several cycles of folding and "proofreading" by

the members of folding and quality control pathway [16]. The oligosaccharide component of the glycoprotein plays a crucial role in this process and also dictates the length of its stay in the folding cycle. Briefly, in this process, the oligosaccharide $Glc_3Man_9GlcNAc_2$ (where Glc is glucose, Man is mannose and GlcNAc is N-acetyl glucosamine), attached to the protein, undergoes trimming of its first two glucose residues by the action of ER resident glucosidases I and II, which results in the formation of $Glc_1Man_9GlcNac_2$. This initiates the calnexin folding cycle, which in mammals involves the ER lectins calnexin and calreticulin that bind the trimmed oligosaccharide on the glycoprotein. Calnexin and calreticulin can work in conjunction with ER oxidoreductases such as PDI (protein disulfide isomerase) and Erp57 that assist in folding by forming correct disulfide bonds [17]. Removal of the third glucose residue by Glucosidase II results in the dissociation of the glycoprotein from calnexin and release from the calnexin cycle. However, until the glycoprotein attains a proper folded conformation, reglucosylation by UGGT (UDP-Glc: glycoprotein glucosyltransferase) may reinitiate the calnexin folding cycle. UGGT presumably recognizes a bipartite signal comprising of the glycan and the distorted polypeptide chain [18]. If the glycoprotein achieves a proper folded conformation, it can no longer be recognized by UGGT and leaves the ER for its destination.

In the event of severe glycoprotein misfolding due to mutations or premature truncation of polypeptide chain, the oligosaccharide undergoes further trimming by ER α-1,2 mannosidases [19]. This leads to the formation of $Man_8GlcNac_2$, which slows down the calnexin folding cycle, due to slower reglucosylation and deglucosylation reactions. Further mannose trimming may generate $Man_6GlcNac_2$ and $Man_5GlcNac_2$ forms and results in the removal of the glycoprotein from the calnexin folding cycle.

Such a misfolded protein is recognized by other ER lectins and escorted out of the lumen for ERAD.

EDEM1 (ER degradation α-1,2 mannosidase like protein) has recently been identified as a soluble ER lectin involved the degradation of misfolded glycoproteins [20]. Subsequent database searches have identified two homologs: EDEM2 and EDEM3 [21,22]. As the name suggests, EDEM1, 2 and 3 bear homology to the ER α-1,2 mannosidases, although their ability to carry out mannosidase activity is controversial. While some reports observe no detectable mannosidase activity of EDEM1, the Molinari group has demonstrated enhanced demannosylation of ERAD substrates upon EDEM1 overexpression [20,23]. In mammalian cells EDEM1, 2 and 3 have been shown to associate and accelerate the turnover of a well-characterized ERAD substrate the NHK (Null Hong Kong) variant of α-1 antitrypsin [24,25]. Subsequently other substrates of the EDEMs have been identified; example, the ricin A chain, and the PiZ variant of α-1 antitrypsin [26,27]. Also, the overexpression of EDEM1, 2 and 3 results in enhanced release of misfolded proteins from the calnexin folding cycle [28,29].

Studies on the yeast homolog of EDEM 1, Htm1p have yielded similar results. Deletion of *HTM1* in budding yeast impaired the turnover of glycoprotein substrates, while no defects in glycan trimming in the quality control cycle were observed [30]. Notably, loss of Htm1p did not have any effect on the turnover of non-glycosylated misfolded proteins such as Sec61-2p (translocon subunit) and ΔGpαF (nonglycosylated yeast pre-pro-α factor), suggesting a function specific to misfolded, glycosylated proteins [30].

Recent work on the subcellular localization of EDEM1 has revealed a novel vesicular transport pathway out of the ER that lacks COPII exit sites [31]. Using electron microscopy staining technique, Zuber and coworkers have demonstrated that EDEM1 is sequestered in budding vesicles along the rough ER cisternae that also contain misfolded glycoprotein substrate (NHK) and other components of the ERAD pathway, namely Derlin-2 (described in detail below). Thus, it appears that the EDEM proteins "escort" the misfolded glycoproteins out of the calnexin folding cycle, and divert them to a pathway out of the ER lumen.

A novel lectin-like protein, Yos9 (Yeast Osteocarcinoma 9), functioning in a similar manner to EDEM has been identified in yeast [32-34]. The lectin-like domain of this protein was found to be homologous to the mannose 6-phosphate receptors. Yos9 has been demonstrated to have preference for glycoprotein substrates containing $Man_8GlcNAc_2$ or $Man_5GlcNAc_2$ N-glycans, suggesting that it acts in the removal of misfolded proteins from the calnexin cycle [35]. Contrary to this finding, another study has reported that Yos9 recognizes unglycosylated substrates and can also function in ERAD despite a mutation in its sugar-binding domain [34]. Since a mammalian homolog of Yos9 has not yet been identified, Yos9 may function in a pathway that is unique to lower eukaryotes such as budding yeast. Unquestionably, more work needs to be done before a clearer picture emerges.

Retrotranslocation of ERAD substrates

After recognition by the ER quality control machinery, aberrant glycoproteins must be transported across the ER membrane to the cytosol for destruction by the 26S proteasome. Simple as it may appear, this process is still an enigma, as the components

of the retrotranslocation channel remain to be conclusively established. Over the course of years, several candidate proteins have been proposed to participate in the formation of the glycoprotein "dislocation pore". These are described below.

The heterotrimeric Sec61 complex performs the translocation of the nascent polypeptide chain into the ER lumen. In yeast this channel is comprised of Sec61p, Sbh1p and Sss1p, while the mammalian subunits include Sec61α, β and γ. The first evidence of the involvement of Sec61 complex in the retrograde export came from studies involving two membrane associated ERAD substrates: major histocompatibility complex class I heavy chains (subsequently referred to as HCs) and the cytomegalovirus (CMV) glycoprotein US2 [36]. It was observed that both of these ERAD substrates associated with the Sec61 complex prior to their degradation. In a separate study, pulse-chase experiments employing yeast strains with mutations in the *sec61* alleles demonstrated a decrease in the ERAD of several substrates such as misfolded yeast carboxypeptidase Y (CPY*) [37].

Recently, the Romisch group has demonstrated that the base of the 19S regulatory particle binds to the Sec61 complex [38]. This interaction of proteasome and the translocon was found to be ATP dependent, suggesting the role of proteasomal ATPase subunits in this process. Additionally, this study also demonstrated that the proteasome could compete with the ribosomes for binding to the Sec61 channel. Moreover, the mutations that disrupted proteasome-Sec61 interaction had no affect on the interactions of ribosome with Sec61 and vice-versa [39]. Collectively these reports indicate that the Sec61 channel may function as a bi-directional channel involved in protein translocation *into* the ER as well as protein dislocation *out* of the ER. If this

indeed were true, it would pose a perplexing problem of regulating protein synthesis along with retrograde export.

The Derlin family of proteins (Derlin-1, Derlin2 and Derlin-3 in humans) constitute a novel class of ER membrane proteins that have been implicated in the retrotranslocation process of misfolded proteins from the ER [40,41]. These small proteins ~22kDa in size, span the ER membrane four times with both their amino and carboxy terminal residing in the cytosol. Sequence analysis indicates that the Derlin proteins are distant orthologs of the yeast Der1p (degradation in ER), sharing just 13% identity. Interestingly, the Der1p was identified in a screen for mutants that caused the stabilization and retention of ERAD substrates (CPY* and PrA*) in the ER lumen **[42]**. A yeast homolog of Der1p, Dfm1 has also been identified, which paradoxically does not seem to be involved in ERAD of Der1 dependent or independent substrates [43]. However, Dfm1 was postulated to function in ER homeostasis, as its deletion or overexpression resulted in the induction of UPR.

The discovery of the Derlin proteins is relatively recent. Derlin-1 was originally identified as an interacting partner of the CMV glycoprotein US11 [40]. Infection of human cells by CMV causes the production of the US11 glycoprotein that disables antigen presentation by HCs. The biochemical process underlying this phenomenon is that the US11 protein produced by the CMV targets HCs for proteasomal degradation. In a screen to identify novel cellular proteins that aid in this process, wild type (WT) and mutant, dysfunctional form of US11 proteins were expressed in U373 cells. Lysates from these cells were then used to search for proteins that specifically associated with WT US11 but not the mutant US11. Such a protein identified from the screen was

Derlin-1. It was observed that the overexpression of GFP tagged Derlin-1 (which presumably functions as a dominant negative form) decreased the turnover rate of HCs.

A separate study that came out in parallel to the aforementioned report showed that the depletion of Derlin-1 by RNA interference in *C. elegans* induced UPR in the organism [41]. In the same study, another novel ER membrane protein VIMP (VCP interacting membrane protein) was identified, that was shown to mediate interaction between Derlin-1 and p97 (valosin containing protein or VCP). P97 is a cytosolic protein that belongs to the class of AAA ATPase family of proteins that aid in the extraction of misfolded glycoproteins from the ER. The role of p97 in the ERAD process will be discussed in detail in a subsequent section.

Since the identification of Derlin-1 in the ERAD process, Derlin-2 and Derlin-3, which function in manner similar to Derlin-1, have been identified [25]. Interestingly, Derlin-2 and Derlin-3 were demonstrated to interact with the ER lectin EDEM1 and were also found to localize in the EDEM1-rich vesicles originating from the rough ER (discussed in a previous section). NHK turnover is accelerated by Derlin-2 and Derlin-3, overexpression, while Derlin-1 overexpression has no effect in this process. Subsequently, the cystic fibrosis transmembrane conductance regulator (CFTR) was identified as a substrate of Derlin-1 **[44,45]**. Thus, there appears to be a trend of substrate selectivity among the Derlins.

Further implication of the Derlins in the process of dislocation comes from the findings that these proteins exist in a complex with other ERAD proteins [46,47]. For instance, Derlin-2 was found to be a part of a complex consisting of the ubiquitin-ligase Hrd1, SEL1 (a novel ERAD protein, Ubx2 in yeast), VIMP and p97. Another study has documented the presence of a complex that is composed of Derlin-1, along with the

ubiquitin domain protein HERP (homocysteine induced endoplasmic reticulum protein), Hrd1, VIMP and p97 [48].

A novel approach to study the process of retrotranslocation *in vitro* was developed by the Johnson group [49]. In this technique, an ERAD substrate ΔGpαF was fluorescence labeled and encased in mammalian ER microsomes, along with a selection of purified luminal ER proteins. The resulting vesicles were mixed with cytosolic proteins under study and the movement of fluorescence labeled ΔGpαF into the cytosol was followed spectroscopically. By sequentially changing the luminal contents of the vesicle and the cytosolic environment, proteins that were involved in the process of retrotranslocation were identified. Two proteins that played a crucial role in this process were PDI and Derlin-1. Clearly, there is growing evidence suggesting a role of the Derlins in the process of retrograde export from the ER. However, the mechanism underlying this process is still very poorly understood. The answer to the question of how these small membrane proteins form a protein-conducting channel still remains elusive.

Signal peptide peptidase (SPP) is an ER membrane-associated aspartic protease that cleaves polypeptides in their transmembrane region. In a screen identical to the one used for the identification of Derlin-1, SPP was revealed as the interacting partner of WT US2 protein [50]. The US2 glycoprotein is also synthesized by CMV, as is the previously mentioned US11 glycoprotein. US2 has also been shown to disable antigen presentation by HCs, again by targeting them for proteasomal-mediated destruction. However, US2 utilizes a pathway different from the US11 protein. It was therefore demonstrated by the use of short hairpin RNA (shRNA), that SPP is responsible for US2 mediated HC dislocation. The implication of this report is that SPP, which spans

the ER membrane seven to nine times, may also be a good candidate for the putative dislocation channel. SPP orthologs do not exist in yeast, thus the SPP pathway may be unique to higher eukaryotes.

Other candidate proteins for the dislocation pore have been proposed. These are: the TEB4 E3-ubiquitin ligase (Doa10 in yeast), which spans the ER membrane thirteen to fourteen times and the TRAP complex (translocon associated protein) [51,52]. The TRAP complex contains four transmembrane ER subunits that associate with the Sec61 channel (another candidate for the retrotranslocon). It was shown that the subunits of the TRAP complex were induced upon ER stress and a knockdown of each of the subunits by RNAi caused delayed degradation of ERAD substrates.

It is important to reiterate that though there are several strong contenders for the dislocation pore forming proteins, there is a lack of concrete evidence establishing the same. Also noteworthy is the idea that there may be several different retrotranslocons that may function specifically for a subset of glycoproteins. This concept may explain the observation that the deletion of Der1p in yeast (or the knockdown of the Derlins in mammalian cells) does not cause cell lethality.

Polyubiquitination of glycoproteins

Ubiquitin (Ub) is a small protein, 76 amino acids in length (~8KDa) that decides the cellular fate of proteins [53]. Addition of Ub to Lys residues of proteins can have several outcomes that range from endocytosis of plasma membrane receptors, lysosomal targeting and proteasome mediated protein degradation [54]. The process of ubiquitination can be broken down into three consecutive steps [55]. First, the Ub is charged on to an Ub-activating enzyme (E1) by the formation of high energy Ub-

thioester intermediate that involves ATP hydrolysis. In the next step, the Ub is transferred to Ub-conjugating enzyme or E2 that again results in the formation of a transient thioester-bonded E2-Ub complex. In the final step, the activated Ub is attached onto the ε-amino group of the Lys residue of the substrate protein, resulting in the formation of an isopeptide bond. The activity of an Ub-ligase or an E3 is crucial for this step. Targeting proteins for proteasomal destruction requires the formation of poly-Ub chains consisting of at least four Ub molecules. This is achieved by the sequential addition of Ub molecules onto Lys-48 residue of the previously attached Ub by repetitive cycles of process described above.

Organization of the Ub conjugating system in organism is hierarchical [55]. There is a *single* E1 that can activate Ub onto a *few* E2s; which can in turn interact with *several* E3s. The E3s can have a wide repertoire of substrate proteins; also a single substrate can be targeted by more than one E3. Such an intricate, intertwined signaling network has evolved to exert a tight regulatory control, as the ubiquitin-proteasome system is involved in critical cellular processes like cell cycle progression, activation of transcription factors and antigen presentation.

E1 enzyme: The E1 is involved in the activation of Ub onto an E2 [56]. This process requires the formation of high-energy thioester between the active site Cys of E1 and the carboxyl group of G76 residue of Ub. The E1 has a site for binding for ATP, the hydrolysis of which is essential to drive this process. It has been proposed that ATP induced conformational change exposes the binding site for Ub on the E1 [57].

As previously mentioned, there is just a single E1 enzyme in most living organisms and that is found to localize in the cytosol and the nucleus. In yeast, the deletion of the *UBA1* gene encoding the E1 results in lethality [58].

E2s: Upon the formation of E2-Ub thioester bond, the E2s catalyze the subsequent transfer of Ub onto the substrate or E3 (HECT E3s, see below). As in the case of E1, the E2 active site also contains a conserved Cys essential for ubiquitination. Additionally, the E2 possess a UBC domain that is involved in mediating interactions with E3s [55].

In yeast there are at least eleven E2s, which function in diverse pathways such as sporulation, peroxisome biogenesis, DNA damage response cell cycle and ERAD. Depending on the function the E2s localize to the nucleus, cytosol or the ER membrane. Mammalian cells have a slightly larger number of E2s. Some of these are expressed in specific tissue (testis specific UBC4), cell type (E2-EPF, keratinocytes) or organelle (BRUCE, Golgi) [59].

E3s. The E3s catalyze the final step of ubiquitination process: the transfer of Ub from an E2 to the substrate. The E3s are divided into two major groups the HECT E3s and the RING E3s.

HECT E3s: The E3s classified under this category contain a segment of ~350 residues that has homology to the E6 associated protein's carboxy terminus (hence the name HECT domain) [60]. The E6 associated protein was first identified as a mammalian protein that associated with the E6 protein produced by the human papilloma virus (HPV). The E6 associated protein was shown to be involved in degradation of p53.

The HECT domain E3s show a fair degree of homology (35-45% identity) in the HECT domain region. This region contains a conserved Cys residue, which is important for thioester bond formation with Ub [61]. The HECT domain is also involved in the interaction of the E3s with the E2s. The N-terminus of the HECT E3s

probably functions in substrate selection. Besides the E6 associated protein, other examples of well-characterized HECT E3s are Rsp5 (yeast) and its human ortholog NEDD4. Rsp5 is localized to cystosol and is involved distinct processes such as endocytosis of plasma membrane permeases and receptors, activation of transcription factors and DNA damage induced ubiquitination of RNA polymerase II [59].

RING E3s: RING (really interesting new gene) finger E3s were originally thought to mediate protein-protein interactions, but later demonstrated to function as Ub-ligases catalyzing the final step of the ubiquitination reaction [62]. Ironically, the RING finger E3s can also catalyze auto-polyubiquitination, thereby regulating their own turnover.

The RING finger domain is characterized by the ability to bind two Zn2+ cations, due the formation of a "crossbrace" resulting from eight conserved Cys and His residues in this region. The RING domain can be sub-classified as RING-HC (His at position 4) or RING-H2 (His at position 4 and 5) and functions in the recruitment of an E2. The crystal structure of the RING domain indicates that it acts as a scaffold, which brings together the E2 and substrate, to orchestrate the transfer of Ub [63].

In recent years, a growing number of ER resident E3s have been identified that are involved in the turnover of misfolded glycoproteins [64]. Hrd1 was the first ER anchored E3 to be identified in yeast that was essential for the turnover of Hmg2p, an isozyme of the sterol biosynthetic enzyme, HMG-CoA reductase [65]. In the same study it was shown that Hrd1 specifically associated with and required the activity of the E2s Ubc7 and Ubc1p for its function. Later it was shown that Hrd1 interacts with the yeast membrane anchored protein Cue1p, presumably to facilitate its function in ubiquitination [66].

Subsequently, the mammalian homolog of Hrd1 was identified that was found to degrade misfolded substrates such as unassembled TCR subunits TCRα and CD3-δ [67]. Notably, the unglycosylated form of prion protein PrP in yeast and the huntingtin protein in mammalian cells were also shown to degrade in a Hrd1 dependent manner [68,69].

The Hrd1 protein is predicted to have five transmembrane domains and interacts with several other proteins of the ERAD pathway. The yeast Hrd1p has recently been shown to be form the core of a complex containing the ER lectin Yos9, (discussed in a previous section), Hrd3 (an ER membrane protein that stabilizes Hrd1), Ubx2 (a UBA and UBL containing protein that recruits Cdc48) and Der1 [70].

Doa10p is a RING-CH type E3 Ub-ligase that is predicted to have fourteen membrane spanning domains [71]. The Doa10p has been shown to localize to the ER as well as the nuclear membrane [72]. Like Hrd1, Doa10p shows dependence for the E2 Ubc7 (and also Ubc6, dispensable in the case of Hrd1) and also the Cue1p protein [73]. Although, unlike Hrd1, Doa10p forms a distinct complex that does not require Der1p [74].

Gp78, also known as autocrine motility factor receptor (AMFR), a 78 kDa type 1 membrane protein, was originally identified in murine melanoma cells and was implicated in tumor cell motility [75]. Elevated levels of AMFR were subsequently correlated with the metastasis of melanoma, lung, colon, oral and bladder cancers, and AMFR overexpression in NIH3T3 fibroblasts was demonstrated to induce their transformation [76-80]. AMFR localizes to the endoplasmic reticulum (ER) and is also believed to be concentrated in the caveolae at the cell surface where its thought to bind it's ligand, autocrine motility factor (AMF) [81]. It has been proposed that the AMF-

AMFR complex is internalized to a smooth sub-domain of the ER from the plasma membrane in a caveolae-dependent manner. The relevance of the AMFR function of gp78 will be examined in detail in Chapter 4.

The function of gp78 as a RING finger E3 Ub-ligase was first demonstrated in a study carried out by the Weissman lab [82]. It was shown that gp78 recruits the cytosolic E2 MmUbc7 (Ube2g2) and targets CD3-δ for degradation. Interestingly, overexpression of inactive gp78 blocked CD3-δ degradation and led to its accumulation in the ER, thereby indicating that ubiquitination of CD3-δ occurs before its extraction into the cytosol. Since its identification as an E3, several misfolded-protein substrates of gp78 have been identified. A recent report has demonstrated that gp78 is also involved in the sterol -regulated ubiquitination of HMG-CoA reductase [83].

Like Hrd1, gp78 is also part of a complex involved in the process of ERAD. Interaction of gp78 with p97 has been previously demonstrated [84]. Recently, gp78 was also shown to be part of complex consisting Derlin-1 and VIMP [47].

Investigation of gp78 domain organization has revealed that in addition to the RING domain, gp78 has a CUE domain that binds Ub and shares similarity with the yeast Cue1p. Additionally, there is also an E2 binding domain (termed G2BR for Ube2g2 binding region), which recruits Ube2g2. Both the CUE domain and the G2BR are essential for gp78 activity [85].

A new perspective of the gp78's role in ERAD was put forth in a study employing the E2, Ube2ge2, and gp78 [86]. Using an *in vitro* assay, it was demonstrated for the first time that Lys-48 linked poly-Ub chains can be preassembled on an E2 (Ube2g2 in this case) and that gp78 can transfer these *en bloc* onto a substrate protein. This is reminiscent of the activity of E4s (see below).

Multi-subunit E3s - SCF complex: Three well-studied multi-subunit E3s are the SCF E3s (e.g., Skp1-Cullin-F-box protein), APC (anaphase promoting complex) and VCB. The SCF complex is important with respect to the ERAD of glycoproteins as this complex consists of the F-box proteins (Fbs1 and 2) that are involved in substrate selection [87,88]. The F-box proteins specifically select misfolded glycoprotein substrate through the recognition of their N-glycan chain. Thus, the SCF complex constitutes a unique and highly specialized class of E3s.

U-box proteins and E4s: U-box proteins belong to a sub-family of E3 ligases that catalyze the elongation of short poly-Ub chains by transferring Ub onto a previously-conjugated Ub molecule [55]. The E4 enzymes catalyze this process in conjunction with E1, E2s and E3s. E4s can also perform ubiquitination of Ub-fusion proteins (stable Ub attached to NH2 terminus of proteins). A classic example of this is the yeast Ufd2 protein. The E4s are characterized by the presence of a U-box, which is similar to the RING domain except that it lacks the ability to chelate metal cations. This is due to the absence of conserved Cys residues that perform metal chelation in the case of the RING domain. The formation of the "U-box" scaffold is presumably attributed to the presence of salt bridges and formation of hydrogen bonds in this domain [89].

CHIP (C terminus of Hsc interacting protein) is an example of a mammalian U-box protein. CHIP has been shown to interact with the cytosolic Hsc70 and catalyze the degradation of the CFTR protein along with Hsc70. Since CHIP lacks a substrate recognition site, it is the Hsp70 that recognizes the misfolded state of CFTR and targets its degradation by CHIP [90]. CHIP has also been shown to work in

coordination with other E3s. For instance, the degradation of Pael receptor is mediated by the combined action of Parkin (E3) and CHIP [91].

Cytosolic-factors of the ERAD pathway

Cdc48/p97: The AAA ATPase, p97 is perhaps one of the most versatile of cellular proteins, for its activity has been illustrated in diverse biological processes such as cell division, membrane fusion and ERAD. The yeast ortholog of p97, Cdc48, was first identified as a protein important for progression through the cell cycle [92]. In the following years, Cdc48 received much attention for its role in protein degradation via the Ub-proteasome pathway. Indeed, virtually all misfolded proteins exiting the ER for proteasomal degradation require p97 function, which "mechanistically drives" the misfolded protein out of the dislocation channel.

Studies on the domain architecture of p97 have provided insights into the functioning of this protein [93]. P97 has an N-terminal domain and two consecutive ATPase domains (D1 and D2 respectively). The N-terminal domain of p97 is required for its interaction with adaptor protein and will be discussed shortly. The two ATPase domains are crucial for p97 function, as the hydrolysis of ATP generates the mechanical force required for various cellular processes. Structural analysis of Cdc48 and p97 has revealed that this protein can exist as a homohexameric "barrel", with the two ATPase domains stacked together forming a thin central pore. Co-crystallization of p97 with different nucleotides and electron microscopy data presented somewhat different opinions of p97 mechanism. The overall indication seems to be that the D1 domain along with the D1-D2 linkers is responsible for the nucleotide-independent formation of the p97/Cdc48 hexamer. The D2 domain on the other hand contributes towards the

p97/Cdc48 ATPase activity. Several reports have indicated that although the D1 and D2 domains are highly homologous and share the same ATPase function, mutations in the D2 domain cause severe defects in ERAD resulting in loss of cell viability. On the other hand, similar mutations in the D1 domain have a less severe impact. Cryo-EM and biochemical studies have shown that the D2 domain undergoes a drastic conformational change upon ATP hydrolysis, while the D1 domain remains stable. However, the D1 domain has been shown to expand during heat treatment, facilitating ATP hydrolysis.

The N-terminus of p97 is involved in binding of its cofactors that mediate recognition of misfolded glycoproteins and target them to the proteasome [94]. These include p47 and Ufd1-Npl4 complex. The N-terminus of p97/Cdc48 was demonstrated to bind directly to polyubiquitinated misfolded proteins. Recently interaction of p97 with the complex of VIMP and Derlin-1 has been described [46,47]. This interaction is at least in part responsible for ER association of p97. Another ER membrane protein Ubx2 has also been shown to recruit Cdc48 to the ER [95].

The C-terminus of p97 is highly flexible and difficult to detect in cryo-EM and crystal structures. At the very end of this domain is a stretch of acidic amino acids that contain a Tyr phosphorylation site (Tyr805). Tyr phosphorylation of Cdc48 is involved in its nuclear export, during the late G-1 phase of the cell cycle [96]. Co-crystallization of the PNGase PUB domain with the C10 peptide (sequence identical to last ten residues of p97) identified the residues important for this interaction [97]. *In vitro* binding studies between C10 peptide and PNGase showed that the phosphorylation of Tyr (corresponding Tyr805 of p97) abrogates this interaction. Curiously, the phosphorylation of this residue also abolished the interaction between C10 and the p97

cofactor Ufd3. This result suggests that phosphorylation of p97 may regulate its function in the ERAD pathway.

Other receptors for Ub: The UBA (Ub-associated domains) are known to bind Ub and therefore function as binding modules for proteins of the ERAD pathway. The UBX (Ub regulatory) domains on the other hand are usually found on the C-terminal regions of proteins and structurally resemble Ub [98]. Proteins containing both the UBA and UBL domains can therefore simultaneously interact with Ub, as well the components of the proteasome, which allows them to form a "bridge" between the misfolded polyubiquitinylated proteins and the proteasome.

Rad23 has been shown to shuttle substrate proteins to the proteasome. The cyclin-dependent protein kinase inhibitor Sic-1's degradation was shown to be Rad23 dependent. This study illustrated Rad23's function of targeting substrates to the proteasome [99]. Other factors such as Dsk2 and Ddi1 have also been identified as Ub receptors, functioning in a similar way as Rad23 [100,101].

Recently a novel Ub-binding protein ZNF216 has been shown to target polyubiquitinated proteins for degradation, by binding Poly-Ub chains via its N-terminal UBD (Ub binding domain). Deletion of *ZNF216* gene in mice led to the accumulation of Poly-Ub substrates in the muscle tissue.

Role of peptide: *N*-glycanase in glycoprotein turnover

Peptide: *N*-glycanase (PNGase) is a deglycosylating enzyme that catalyses the removal of N-linked glycans from glycopeptide and glycoprotein substrates. Although PNGase was first isolated from bacteria over ten years ago, identification of the gene encoding the yeast PNGase (Png1) has marked the beginning of extensive research on

structure and physiological properties of this enzyme in eukaryotes [102]. The *PNG1* gene encodes for the cytosolic protein Png1p, which was originally thought to act on glycopeptides (but subsequently shown to act on full-length glycoproteins). This finding was of great significance, for it implicated the existence of a pathway for the removal (and recycling) of N-glycans upon glycoprotein degradation. The subsequent identification of Rad23 (a Ub adaptor protein) as the interacting partner of Png1 emphasized the involvement of PNGase in the ERAD pathway [103,104].

Sequence analysis of Png1 shows that it belongs to the transglutaminase family of proteins, which catalyze amide bond formation between the γ-carboxyamide group of glutamine, and the ε-amino group of lysine [105]. PNGase catalyzes the opposite reaction i.e., the cleavage of amide bond between the Asn and the GlcNAc of the glycan. Site directed mutagenesis study of Png1 has revealed that the conserved catalytic triad of Cys191, His218 and Asp235 in the transglutaminase domain is essential for Png1 activity and has been proposed to form the enzyme's active site. In agreement with this hypothesis, mutation of any of the catalytic triad residues abolishes N-glycanase activity [105].

Database searches have revealed the presence of PNGase genes in other eukaryotes such as *Caenorhabditis elegans*, *Drosphila melanogaster*, *Mus musculus* and *Arabidopsis thaliana* [106]. The organization of PNGase in these organisms differs from Png1. While Png1 has very short amino (N) and carboxy (C) terminal domains, The N and C terminal domains of other species are significantly larger. The N-terminal domain of *C. elegans* has a thioredoxin domain, and was recently shown to play a dual role of an oxidoreductase as well as an N-glycanase [107]. The mouse and fruit fly PNGase have a unique protein-protein interaction motif, the PUB domain (peptide: N-glycanase and

UBA/UBX containing proteins) [108]. In *A. thaliana*, PNGase has four homologs, which have the PUB domain as well the UBA or the UBX domains.

The PUB domain of mouse PNGase was shown to bind the S4 proteasomal subunit via HR23B [104]. A part of the N-terminal region of mouse PNGase, and its middle domain have been demonstrated to participate in binding to mouse HR23B, an ortholog of the yeast Rad23 [104]. HR23B is a DNA repair enzyme and was shown to stabilize the Xeroderma pigmentosum C (XPC) protein by binding to it during DNA damage repair. The binding region on HR23B has thus been termed XPCB. The interaction between Png1 and Rad23 in yeast has been shown to occur via the XPCB domain of Rad23, although data from the crystal structure of Png1 in complex with Rad23 suggests that other regions on the N- and C- terminal parts of Png1 strengthen this interaction [109]. The crystal structure of the complex of core domain of mouse PNGase and HR23B was also solved and showed that the interaction of these two proteins is mediated via the XPCB domain of HR23B, albeit through different regions of mouse PNGase [110]. The significance of the yeast Rad23 and Png1 complex was highlighted by the finding that the efficient degradation of ricin A toxin is dependent on the formation of a functional complex between Rad23 and Png1 [111].

Recently, the C-terminal region of p97 was also shown to bind to the PUB domain of mPNGase [112]. P97 has also been shown to interact with an ER resident E3 Ub-ligase gp78. Coimmunoprecipitation analysis of lysates from Cos-1 cells indicates that PNGase is a part of the complex consisting of gp78 and p97 [113]. Direct interaction between PNGase and gp78 has not been observed. As discussed in a prior section, Tyr phosphorylation of p97 has been proposed to block PNGase interaction. Finally, the PUB domain of PNGase was also demonstrated to interact with the C-

terminus of Derlin-1 [114]. The significance of this interaction will be addressed in Chapter 3 of this dissertation.

Combining all the aforementioned observations has led to the postulation of the presence of an ER associated "degradation complex". Such a degradation complex consists of Derlin-1 that recruits a sub-complex consisting of PNGase, p97, gp78 and HR23B. HR23B forms the link to the proteasome. Alternatively, the proteasome can S4 directly bind the PNGase subcomplex via its S4 subunit.

As mentioned before, PNGase was previously thought to act only upon glycopeptides [102]. It was therefore presumed that PNGase action occurred after the degradation of the glycoprotein by the proteasome. The fact that PNGase does indeed act upon full-length glycoproteins was first demonstrated in COS cells. By inhibiting the activity of the proteasome, it was demonstrated that deglycosylated products accumulate in the cytosol, indicating that PNGase can act prior to glycoprotein degradation [115]. Further, by employing siRNA directed towards mammalian PNGase, it was shown that the deglycosylation activity in COS cells could be inhibited. This observation proved that the deglycosylated intermediates that were produced upon proteasomal-inhibition were due to PNGase action on glycoproteins. Subsequently, studies on HC degradation showed that treatment with siRNA directed towards PNGase had no effect on the dislocation of HC from the ER to the cytosol [116]. This result indicated that PNGase activity was not required for the process of retrotranslocation.

Interestingly, PNGase can distinguish between non-native, versus native glycoprotein substrates. This unique characteristic of PNGase has been demonstrated

using the misfolded PiZ variant of α-1antitrypsin, chemically denatured RNase B and heat denatured CPY and ovalbumin respectively [117,118].

PNGase has been demonstrated to have a preference for high-mannose type N-glycans. This property was evaluated in a recent study involving the co-crystallization of mouse PNGase with mannopentaose [119]. The study uncovered a glycan-binding region in the C-terminal of mouse PNGase, which is structurally similar to the sugar-binding domain of Fbs1 (a component of the SCF Ub-ligase).

To aid in the study of physiological role of PNGase in living cells, a PNGase inhibitor, Z-VAD-fmk was developed. Z-VAD-fmk was originally shown to function as a caspase inhibitor but in low concentration it was shown to irreversibly inhibit PNGase activity [120]. This drug has been employed in a co-crystallization study of yeast Png1 and was also used for biochemical analysis of PNGase from mammalian cells. Subsequently, haloacetoamidyl derivatives of N-glycans were designed that target the active site Cys of PNGase and thus, function in a similar manner to z-VAD-fmk [121].

Proteasome: the cell's machinery for protein destruction

The 26S proteasome is a multi-subunit assemblage, consisting of a 20S core-complex and a 19S regulatory particle [122,123]. The 20S core particle is a barrel-shaped structure and consists of four stacked heptameric rings. The rings of the 20S core are composed of two central β-rings and two distal α-rings, each in turn composed of seven distinct subunits. The proteolytic activity of the 20S core is contributed by only three subunits of the β-ring, namely β1, β5 and β7. These subunits line the lumen of the 20S barrel and therefore access the substrate polypeptide-chain "threading" through the cylinder. The α-subunits on the other hand are involved in controlling the

movement of the substrate through the 20S core and the removal of degradation products out of the core. Despite possessing the catalytic activity, the 20S subunit of the proteasome is latent on its own. Complex formation between the 20S and 19S particle is essential for the initiation of proteolytic activity. Thus, the 19S particle is also known as the regulatory particle.

The 19S (also known as PA700) proteasomal subunit consists of a base and a lid. The base of the 19S consists of six ATPase subunits and two non-ATPase subunits and is always in direct contact with the 20S core particle. The lid, on the other hand, sits on top of the base and this connection between the two is mediated by Rpn10 subunit.

As mentioned in a previous section, formation of a poly-Ub chain of four or more covalently linked Ub is essential for targeting of the substrate to the proteasome. It has been demonstrated that the 19S lid functions in the recognition of polyubiquitinated substrates. This recognition is mediated by the Rpn10 subunit. Another report has suggested that Rpt5 is also involved in this process. While the polyubiquitinated substrate remains attached to the lid, via the Ub, ATPase subunits lead the other end of the polypeptide into the 20s pore. ATPase activity of Rpt2 causes gating of the channel, causing expansion of the core and thereby allowing accessibility of the polypeptide into the core. Upon the entry of the polypeptide into the 20S core, the de-ubiquitinating enzyme Rpn11 hydrolyzes the isopeptide bond between the polypeptide and Ub, thereby causing the release of the poly-Ub chains. Some reports have also indicated that the 19S lid has an unfoldase activity, that allows for an easier passage into the 20S core.In higher eukaryotes, two other regulatory particles for the 20S core have been described [124]. These are PA200 and PA28. Like the 19S particle, the PA28 particle is also multi-subunit, while the PA200 is a single chain protein. Both, PA200 and PA28 can

form mixed proteasomes in which the 20S core is bound by PA700 at one end and PA200 (or PA28) at the other end. Although, not much is known about the significance, these have been proposed to function in the generation of antigenic peptides by the immune cells.

Chapter 2. Misfolding is a Prerequisite for Peptide: *N*-Glycanase Mediated Deglycosylation

Summary

Peptide: *N*-glycanase (PNGase) is a deglycosylating enzyme that catalyzes the hydrolysis of the β-aspartylglycosylamine bond of aspargine-linked glycopeptides and glycoproteins. Earlier studies from our lab indicated that PNGase catalyzed de-*N*-glycosylation was limited to glycopeptide substrates, but recent reports have demonstrated that it also acts upon full-length misfolded glycoproteins. In this study we utilized two glycoprotein substrates, yeast carboxypeptidase (CPY) and chicken egg albumin (ovalbumin) to study the deglycosylation activity of yeast PNGase and its mutants. Our results provide further evidence that PNGase acts upon full-length glycoprotein substrates and clearly establish that PNGase activity is specific for misfolded or denatured glycoproteins.

Materials and methods

1. Plasmid constructs and reagents:

Cloning of yeast Png1 and generation of its mutant constructs in PET28a$^+$ expression vector has been previously described [105]. Yeast carboxypeptidase was purchased from Roche Diagnostics, Indianapolis, IN and ovalbumin from Sigma, St. Louis, MO.

2. Glycoprotein denaturation:

Stock solutions of CPY and ovalbumin were prepared in distilled water at the concentration of 2 mg/mL. To carry out glycoprotein denaturation, an aliquot from the stock of CPY or ovalbumin was heated at 100°C for 20 minutes and rapidly frozen in dry ice-ethanol bath.

3. Circular dichroism analysis:

All circular dichroism measurements of native as well as denatured CPY and ovalbumin were performed on Jasco J-715 spectropolarimeter. A 1 cm path length cell was used to collect data in the far UV range. Sample concentrations of 4.0 μM of CPY and 2.6 μM of ovalbumin were used to collect data in the far UV. Spectra were collected with a scan rate of 100 nm/min, with a 1 nm bandwidth, and a 1 sec response time. Spectral measurements were performed in distilled water and the spectrum of a "blank" sample containing distilled water was subtracted from all sample values.

4. Protein expression and extraction:

Escherichia coli BL21 (DE3) pLysS cells were transformed with $(His)_6$-tagged wild type Png1p, or its mutant constructs (C165S, C191A, R210A, H218A, W220A, W231A and

D235A). Protein expression was performed by inducing 50mL culture with 1mM isopropylβ-D-thiogalactosidase (IPTG) at A_{600} = 0.6. After 4 hrs incubation at 30°C, the cells were harvested at 3000 X g for 20 min. Protein extraction was performed by adding 3 mL of lysis buffer comprised of phosphate buffer saline, 1% Triton X-100, 1mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF). The mixture was subjected to sonication on ice, using a Branson sonicator at level 3, for five 30 sec periods with intermittent 30 sec cooling intervals. The resulting cell extract was centrifuged at 16,000 X g for 10 min at 4°C and the supernatant obtained was used for the deglycosylation assay.

5. Deglycosylation assay:

A reaction mixture containing 40 μL of *E. coli* lysate expressing wild type Png1, its mutant or expression vector alone (control) was incubated with 80μg of CPY or ovalbumin in 1 mL of buffer A (5 mM DTT, 20 mM Na_2HPO_4, 0.5 M NaCl, pH 7.2) at 25°C for 2 hrs. The deglycosylation reaction was stopped by boiling in SDS-PAGE sample buffer at 100°C for 5 min. Samples of deglycosylation reaction were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with 1:4000 dilutions of rabbit polyclonal anti-ovalbumin antibody (Research Diagnostics Incorporated, Flanders, NJ) or polyclonal anti-CPY antibody (a kind gift from Dr. Neta Dean, department of biochemistry and cell biology, Stony Brook University), followed by a 1:3000 dilution of goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Roche Molecular Biochemicals, Indianapolis, IN). Blots were visualized using the chemiluminescence detected by exposure to medical X-ray film (Fuji photo film Co., Stamford, CT).

Results

1. Heat-Denaturation of CPY Results in the Loss of Secondary Structure:

Heat treatment is a widely employed method for protein denaturation. While some proteins have the propensity to refold upon slow cooling, others assume a non-native or a misfolded state. We therefore exploited the methodology of thermal denaturation to obtain misfolded forms of CPY and ovalbumin. To assess misfolding, comparison of the native and heat-treated CPY or ovalbumin was carried out by circular dichroism. Far UV CD spectra of native and denatured samples of CPY and ovalbumin were measured. As shown in Figure 2.1A, native CPY showed a negative peak at 210nm and a positive peak at 194nm, which is typical for a protein that has a predominant α-helix conformation. In contrast, the spectrum of the denatured CPY showed a low α-helix content, confirming the loss in secondary structure after heat treatment. Native ovalbumin shows a negative peak at 221nm and a positive peak at 192 nm. Although thermally denatured ovalbumin did show some loss in the secondary structure, the effect of heat treatment was not as dramatic as that for CPY (Figure 2.1B).

We ruled out the possibility of insoluble protein aggregates resulting from the harsh heat treatment by performing ultracentrifugation and protein estimation of the denatured protein in the supernatant (data not shown).

2. Wild-type yeast Png1p can deglycosylate heat-denatured CPY but not its native form:

To test for the ability of native and denatured CPY to be deglycosylated, an *E. coli* lysate expressing Png1p was incubated with equal concentrations of native or heat

denatured CPY. Western Blot analysis of deglycosylation reaction using anti-CPY antibody was performed to analyze the deglycosylation of CPY with Png1p. While Png1p had no effect on the native CPY (Figure 2.2, lane 1), complete deglycosylation of denatured CPY by wild type Png1p was observed (Figure 2.2, lane 2) demonstrating a requirement for the misfolded form. No deglycosylation was observed on native or denatured CPY incubated in the absence of Png1p (Figure 2.2, lanes 3 and 4). As a control for deglycosylatyion, we incubated CPY with Endo H, a bacterial *N*-glycanase and observed a similar shift compared to Png1p deglycosylated CPY (Figure 2.2, lane 5). To further compare the deglycosylation pattern of *in vitro* deglycosylated CPY, we analyzed the lysate from wild-type *Saccharomyces cerevisiae* containing endogenous glycosylated CPY (Figure 2.2, lane 6) with the lysate from a yeast mutant of Stt3p (a subunit of oligosaccharyl transferase) defective in N-glycosylation (Figure 2.2, lane 7). In the latter case, CPY undergoes partial glycosylation and as expected, this sample migrates at an intermediate position between fully-glycosylated and deglycosylated forms of CPY.

3. Catalytic triad mutants of Png1p fail to exhibit any deglycosylation activity:

A site directed mutagenesis study of Png1p has implicated the triad of amino acids Cys-191, His-218 and Asp-235 as key components of the catalytic site [105]. We therefore tested the activity of these mutants on denatured CPY and ovalbumin (Figure 2.2 and 2.3 respectively). As expected, the active-site mutants of Png1p show no deglycosylation activity towards heat denatured CPY or ovalbumin (Figure 2.2, lane 8-10 and Figure 2.3, lanes 4-6). In case of ovalbumin, a control of denatured ovalbumin lacking Png1p was loaded (Figure 2.3, lane1) along with native and denatured

ovalbumin incubated together with Png1p (Figure 2.3, lanes 2 and 3). In lane 7 Endo H treated ovalbumin was loaded as a positive control. Interestingly, we observed only a partial deglycosylation of ovalbumin by Png1p, which is in agreement with the results of our CD analysis where we observe only a partial loss of secondary structure (Figure 2.3, lane 3). A doublet seen in the case of ovalbumin probably is indicative of its phosphorylated variants (Figure 2.3).

Discussion

The involvement of PNGase in the ER associated degradation of misfolded glycoproteins had been proposed earlier [106]. However, this became somewhat questionable when it was shown that PNGase acted only on glycopeptides presumably arising from proteasome-mediated proteolysis of glycoproteins [102]. Studies by Suzuki *et. al.* on yeast PNGase corroborated this theory, as it was demonstrated that PNGase could readily deglycosylate glycopeptide substrates with short amino acid backbones and not full length glycoproteins [102,125].

The first evidence of PNGase activity on full-length glycoproteins came from recent *in vivo* studies by Hirsch *et. al.*, who utilized ERAD substrates such as TCRα, and HCs [115,116]. These results were further substantiated by a knockdown of deglycosylation observed in cells treated with siRNA directed towards human PNGase [115,116]. It seems likely that the earlier finding that PNGase is unable to deglycosylate full-length glycoproteins was due to fact that the substrates used in this study were not completely denatured. We now know that the N-glycanase activity is specific for denatured or misfolded polypeptides [117].

Several years ago, our lab reported that DNA repair enzyme Rad23p interacts with PNGase in yeast, and this interaction was further explored in a recent study [103,126]. The mouse homolog of yeast PNGase was also found to interact with the mammalian counterpart of Rad23p, HR23b, Derlin-1, p97 and S4 [127]. These proteins have been identified as important components of the ERAD pathway. It has also been demonstrated by immunofluoresence and subcellular fractionation that human PNGase is associated with the ER via Derlin-1 (discussed in the next chapter), forming a

complex with HR23B, p97 and S4 in HeLa cells [128]. Thus, all the data so far indicates PNGase involvement in ERAD and leads to a model in which a retrotranslocated, misfolded glycoprotein is first deglycosylated in the ER or the cytosol by PNGase and subsequently degraded by the proteasome. We, however, cannot rule out the possibility that in case of some substrates deglycosylation may occur after proteasomal degradation.

Although it has been reported previously that PNGase acts on misfolded polypeptides, our current work on PNGase focuses on the subject of substrate specificity. We addressed this issue by carrying out thermal denaturation of glycoproteins to generate their non-native forms, as opposed to chemical denaturation for the simple reason that many proteins regain their native conformation upon removal or dilution of chemical perturbants such as guanidine hydrochloride or urea. We assessed the unfolded state of CPY and ovalbumin by CD analysis. Extensive misfolding of CPY was observed. In contrast we found that ovalbumin had the tendency to refold, even upon rapid cooling after heat treatment. Such behavior of ovalbumin has been previously described [129]. Incubation of PNGase with native and heat denatured CPY resulted in deglycosylation of the denatured but not native CPY. When heat denatured ovalbumin was used as a substrate partial deglycosylation was observed, which is consistent with the CD analysis indicating the partial refolding of ovalbumin after cooling. These findings therefore demonstrate that misfolding of glycoproteins is a prerequisite for PNGase mediated deglycosylation and that complete glycoprotein unfolding is necessary to achieve deglycosylation.

In summary, we have made use of two additional, novel glycoprotein substrates to study de-*N*-glycanase activity of PNGase. Although our results suggest that PNGase

has a broad range of substrate specificity, it is clearly evident that PNGase demonstrates a high stringency for misfolded versus the native form of glycoproteins.

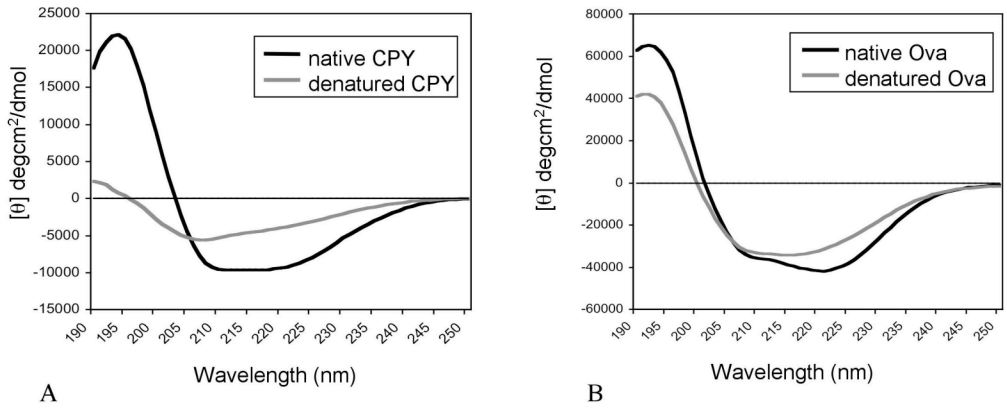

Figure 2.1 Far UV CD spectral analyses of CPY and ovalbumin: Panel A, native and denatured CPY; panel B, native and denatured ovalbumin.

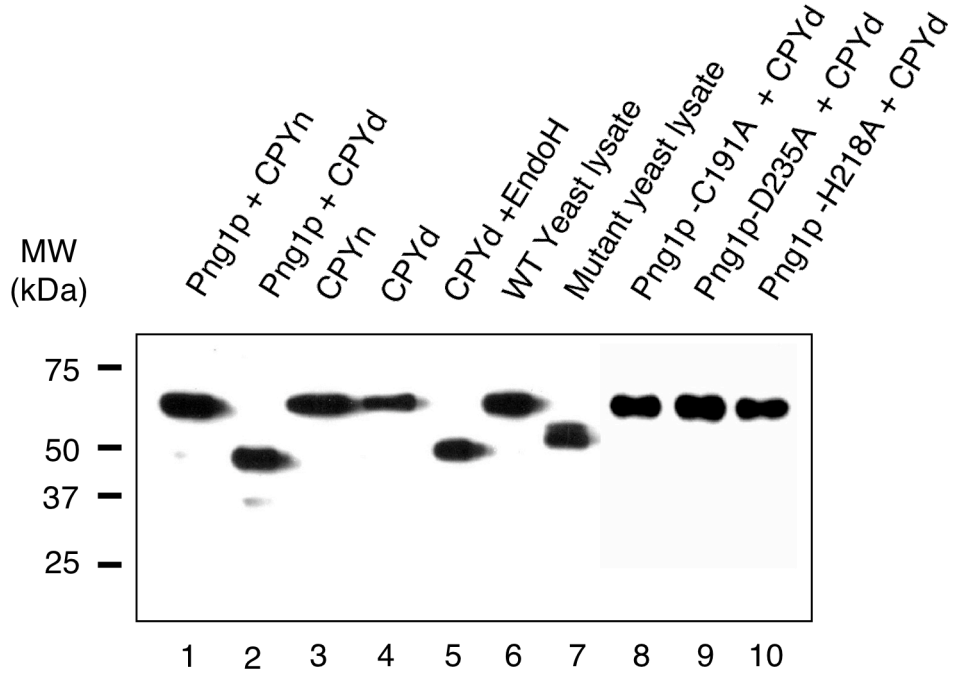

Figure 2.2. Deglycosylation activity of Png1p is specific for misfolded glycoproteins; Png1 catalytic triad mutants do not deglycosylate denatured CPY: Western blot analysis of deglycosylation reaction using CPY antibody. Lane 1, Png1p incubated with native CPY; 2, Png1p incubated with denatured CPY; 3, native CPY alone; 4, denatured CPY alone; 5, CPY treated with Endo-H (bacterial *N*-glycanase); 6, endogenous CPY in a wild-type *S. cerevisiae* lysate; 7, CPY in a yeast lysate from a Stt3 mutant defective in glycosylation; 8, 9 and 10, denatured CPY incubated with active site mutants C191A, D235A and H218A, respectively, of Png1p.

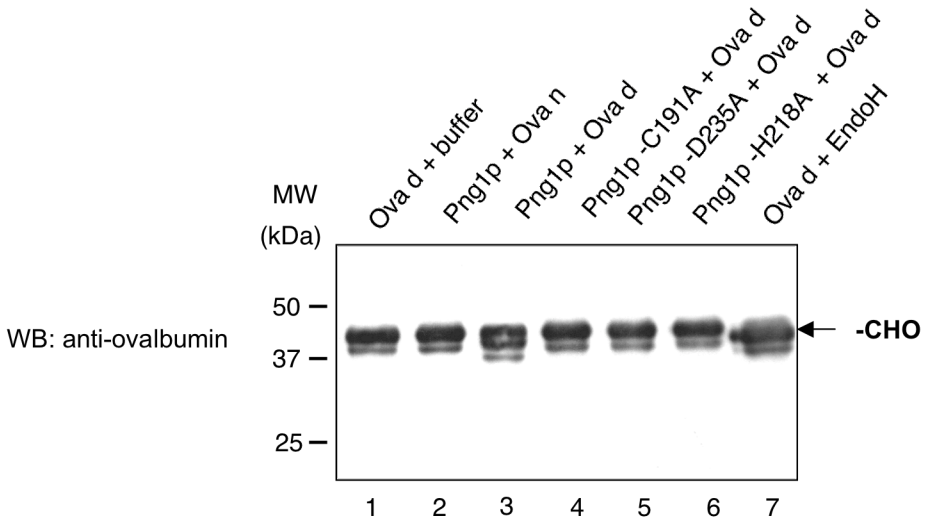

Figure 2.3 Png1p catalytic triad mutants do not deglycosylate denatured ovalbumin: Western blot analysis of deglycosylation reaction using anti-ovalbumin antibody. Lane 1, ovalbumin alone; 2, Png1p treated native ovalbumin; 3, Png1p treated denatured ovalbumin; 4, 5, and 6, active site mutants of Png1p incubated with denatured ovalbumin; 7 ovalbumin treated with Endo H (bacterial *N*-glycanase). –CHO indicates deglycosylated forms of ovalbumin.

Chapter 3. Peptide: *N*-glycanase Associates with the ER via Derlin-1

Summary

Several prior reports have demonstrated the presence of deglycosylated glycoproteins in the cytosol as well as the ER membrane. As discussed in previous sections, the presence of cytosolic PNGase activity has been established beyond reasonable doubt. A recent study demonstrated that a small amount of PNGase associates with the ER membrane, thus implying that deglycosylation can proceed as glycoproteins are exiting the ER. However, the mechanism of PNGase association with the ER membrane remains largely unknown, as PNGase lacks the necessary signal to facilitate its incorporation in the ER membrane, nor is it known to bind an integral ER protein.

Using coimmunoprecipitation analysis, we have identified a membrane protein that associates with PNGase in HeLa cells. This protein, Derlin-1, was recently proposed to mediate the retrotranslocation of misfolded glycoproteins. In this study we demonstrate that Derlin-1 interacts with the N-terminal domain of PNGase via its cytosolic C-terminus, thereby bringing it in close proximity to the ER, and thus providing accessibility to the dislocating glycoproteins.

Materials and Methods

1. Plasmid constructs: The cDNA of human Derlin-1, cloned in a pEGFP vector, was a gift from Dr. Hidde Ploegh (Harvard Medical School, Boston). PCR reactions were performed to generate various fragments (Δ187-251, Δ1-16, Δ1-154, Δ1-187, Δ44-251) having 5′ *BamH*1 and 3′ *EcoR*1' sites, which were subsequently cloned into pcDNA4.0 vector, with a c-terminal Myc tag. Full length Derlin-1 was also cloned into pcDNA 4.0 vector using 5′ *EcoR*1 and 3′ *Xho*1 sites. To synthesize mouse PNG1-GFP fusion plasmid for expression in mammalian cell culture, the mPNG1 cDNA was amplified by PCR to introduce a 5′ *EcoR*1 and 3′ *Sal*1 site. After digestion and purification, full-length mPNG1 was sub-cloned into a pEGFP-C1 plasmid (CLONTECH Co., Palo Alto, CA) to generate a C-terminal GFP fusion. Truncation constructs of mPNG1 (ΔMC, ΔC, ΔNC, ΔN, ΔMN, see Fig. 5A) were also sub-cloned into pEGFP-C1 vector. Full-length mPNG1 and truncations (ΔMC, ΔC, ΔNC, ΔN, ΔMN) were also sub-cloned into a pcDNA3.0 vector with an HA-tag at the C-terminus. The HA-tag (HA_3) was introduced in pcDNA3.0 using 5′ *Not*1 and 3′ *Xho*1 sites. Truncation constructs obtained after PCR reactions using 5′ *EcoR1* and 3′ *Not1* were ligated into pcDNA3.0 containing a HA-tag at the C-terminus.

Histidine-tagged mPNG1 (mPNG1-His_6) and ΔMC-mPNG1-His_6 constructs for *Escherichia coli* expression in pET-28a(+) were made as described previously [127]. A GST-Derlin-1-C fusion plasmid was constructed to have the C-terminal 64 amino acids of Derlin-1 fused to glutathione *S*-transferase (GST) at its N-terminus in order to carry out *in vitro* GST pull-down assays.

2. Mammalian Cell Culture and Transfection: HeLa (R19) cell monolayers were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO-BRL, Rockville MD) supplemented with 10% bovine calf serum (BCS) and 100μg/ml each of penicillin and streptomycin (GIBCO-BRL, Rockville MD). The cells were maintained at 37°C in a 5% CO_2, 95% air atmosphere. Plasmid transfections were performed using lipofectamine 2000™ reagent (Invitrogen, Carlsbad CA), in DMEM supplemented with 10% BCS without antibiotics, according to the manufacturer's protocol.

3. Immunoprecipitation and Immunoblotting: Immunoprecipitations were performed with 2 x 10^7 cells. Twenty-four hours after transfection, cells were harvested, washed with PBS (GIBCO, Rockville, MD) and lysed in digitonin buffer (1% digitonin, 25 mM Tris.HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ with 1 mM PMSF and protease inhibitor cocktail from PIERCE, Rockford, IL) for 1 h at 4°C. The cell lysate was centrifuged for 15 min. at 14000 rpm. The supernatant obtained was incubated with desired antibody for 15 min at room temperature followed by the addition of protein A/G agarose beads as required. This mixture was then incubated overnight at 4°C on a rocker. Subsequently, the beads obtained from the immunoprecipitation reaction were washed three times in buffer containing 0.2% digitonin, 10 mM Tris.HCl, pH 7.4, 150 mM NaCl and 5 mM EDTA. Immune complexes formed on the beads were eluted with 2 X SDS-PAGE sample-buffer and analyzed by SDS-PAGE followed by immunoblotting. Most of the antibodies used in this study were obtained from commercial sources. Mouse monoclonal anti-GFP and anti-HA antibodies were purchased from CLONTECH, Inc. (Palo Alto, CA), mouse monoclonal anti-calnexin; mouse monoclonal anti-α-tubulin and rabbit polyclonal anti-GFP antibodies were obtained from Abcam (Cambridge, MA). Rabbit polyclonal anti-HA, anti-His and anti-Myc antibodies (polyclonal and monoclonal, respectively) were purchased from Santa

Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-calnexin was a gift from Dr. Deborah Brown (State University of New York at Stony Brook, NY). For immunoprecipitation analysis antibodies were used at a dilution of 1:500, while for immunoblotting 1:3000 dilution was used for primary as well as HRP-conjugated secondary antibodies.

4. Immunofluorescence: Hela cells were cultured on coverslips and transfected with appropriate plasmids. 24 hrs post-transfection, cells were fixed in 3% paraformaldehyde for 20 min. at room temperature and permeabilized in 0.5% Triton X-100 on ice for 7 mins. Cells were washed in phosphate buffered saline (PBS) plus 0.5% normalized goat serum (NGS) and incubated with the primary antibodies as indicated (anti-HA 1:200 dilution; anti- calnexin 1:100 dilution). AF-488 or AF-555 conjugated secondary antibodies were used as required. Nuclear DNA was stained with 4', 6-diaminidino-2-phenyindole (DAPI). Cells were analyzed using Zeiss Axiovert confocal microscope (Zeiss, Thornwood, NY) to visualize the proteins under study.

5. Overexpression and Purification of constructs: mPNG1-$(His)_6$ and ΔMC-mPNG1-$(His)_6$ constructs were transformed into *E. coli* BL21(DE3)pLysS cells. The cells were grown in LB media at 37°C and expression was induced by adding 1mM isopropyl β-D-thiogalactoside (IPTG) when the cells reached an absorbance (A_{600}) of 0.6-0.8. After 3h of induction at 30°C the cells were harvested and resuspended in lysis buffer containing 1 X PBS, 1% Triton X-100, 10mM β-mercaptoethanol (β-Me), and 1mM phenylmethylsulfonyl fluoride (PMSF) followed by sonication on ice using a Branson sonicator. The cell extract was centrifuged and supernatant was allowed to pass through chelating Sepharose column obtained from Amersham Biosciences, Inc. (Piscataway, NJ) charged with Ni^{++}. Subsequent

purification was performed according to the manufacturers protocol. Purity of the eluted fractions was analyzed by SDS-PAGE.

GST-Derlin-1-C was transformed in *E. coli* DH5α cells and expression was induced by addition of 0.1mM (IPTG) to the culture. After induction at 30°C for 3h cells were lysed in buffer containing 1 X PBS, 1% TritonX-100, 10mM βMe and 1mM PMSF. The lysate was centrifuged at 14,000 rpm and the supernatant was added to glutathione agarose beads. After 2h incubation at 4°C, the beads were washed three times with the lysis buffer. The bound protein was then eluted in buffer containing 20mM reduced glutathione (GSH) in 50mM Tris-HCl, pH 8.0. The same elution conditions were used to purify GST alone. Pure protein was analyzed on SDS-PAGE. Eluted proteins were subjected to buffer exchange (1 X PBS, 10mM βMe and 1mM PMSF) using a Centricon from Millipore Corp., (Billerica, MA).

6. *In vitro* Binding Assay: Pure GST alone or GST-Derlin-1-C fusion protein extracts (1ml) were incubated with 30 µl (bed volume) of GSH-agarose beads for 1h in binding buffer (1 x PBS, 1% TritonX-100, 10mM βMe and 1mM PMSF) at room temperature, washed five times with binding buffer and incubated with full length mPng1p-His_6 or ΔMC-mPng1p-His_6 in binding buffer for 1h at RT. Beads were washed five times in binding buffer followed by elution of the bound proteins with SDS-PAGE sample buffer. Eluted proteins were resolved on SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with polyclonal anti-His or monoclonal anti-GST antibody.

7. Subcellular Fractionation and PNGase Activity Assay: Subcellular fractionation was performed on a sucrose gradient with 5 x 10^7 HeLa cells after lysing in lysis buffer as

described earlier [130]. The ER membranes sedimenting at the interface between1.2 and 1.5M sucrose layers were collected, washed, pelleted and resuspended in 1 ml digitonin lysis buffer containing (1% digitonin, 25mMTris.HCl, pH 7.4, 150mM NaCl, 5mM $MgCl_2$ with 1mM PMSF and protease inhibitor cocktail) for 1h at 4°C and subjected to immunoprecipitation with anti-HA and anti-GFP antibodies to detect the distribution of PNGase and Derlin-1 in HeLa cells. Immunoprecipitation was also performed on the microsome-free cytosolic fraction and total cell lysate obtained from 5 x 10^7 cells in digitonin lysis buffer (1 ml). Immune complexes were recovered from digitonin lysates using Protein A/G agarose beads and were washed in 0.2% digitonin in 10mM Tris.HCl, pH7.4, 150mM NaCl, 5mM EDTA. Immune complexes were resolved on SDS-PAGE, transferred to nitrocellulose and probed with the specified antibody. Subcellular fractions were also subjected to Western blotting with anti-calnexin (mouse monoclonal) as ER marker and anti-α-tubulin (mouse monoclonal) as cytosolic marker.

The total cell lysate (10μL), microsome free cytosol (10μL) and ER membranes (10μL) were assayed for PNGase activity using a fetuin derived asialoglycopeptide I ([^{14}C]CH3)$_2$Leu-Asn(GlcNAc$_5$Man$_3$-Gal$_3$)-Asp-Ser-Arg) as substrate as previously described [125,131]. Radioactivity was monitored on a Phosphorimager (Molecular Dynamics) and quantitated by using ImageQuant (version 1.2).

Results

1. A membrane receptor for PNGase: Based on biochemical analysis as well as enzyme assays, several previous studies have reported that at least a small amount of PNGase is associated with the ER. PNGase has been shown to participate in the deglycosylation of HCs and a recent report revealed the presence of deglycosylated HCs in the cytosol as well as the ER membrane [40]. This observation raised the possibility that the deglycosylation was mediated by membrane associated PNGase. However, PNGase lacks the signals expected to be necessary for its interaction with the ER.

A novel protein, Derlin-1, has been implicated in the dislocation process of HCs from the ER to the cytosol. Derlin-1 is an ER membrane protein, predicted to have four transmembrane segments, with both its N and C-termini in the cytosol. To explore the possibility that Derlin-1 mediates the association of PNGase with the ER, we studied the interaction of mouse PNGase with human Derlin-1. Mouse homolog of PNGase was used for our studies, since mouse and human PNGase are highly conserved proteins [102]. To analyze a possible interaction between PNGase and Derlin-1, HeLa cells were co-transfected with pcDNA 3.1 vector and Derlin-1-GFP (Figure 3.1, lane 1), pEGFP vector alone and PNGase-HA (Figure 3.1, lane 2) or PNGase-HA and Derlin-1-GFP (Figure 3.1, lane 3). The transfected cells were lysed in 1% digitonin buffer and the total cell lysate was subjected to immunoprecipitation with monoclonal anti-HA antibody or polyclonal anti-GFP antibody. The samples obtained from immunoprecipitation were analyzed by SDS-PAGE followed by immunoblotting using antibodies indicated in Figure 3.1A. The results in Figure 3.1A, lane 3, show that PNGase and Derlin-1 co-precipitate. No precipitation was observed with each of the vectors alone (lane 1 and 2). In a previous report GFP-tagged Derlin-1 was used as a

dominant negative construct based on the fact that the addition of a folded domain to a flexible cytoplasmic tail of Derlin-1 would interfere with interactions on the cytoplasmic face of ER and consequently would block dislocation of HCs from the ER to the cytosol [40]. To rule out the possibility of any non-specific interaction of PNGase with Derlin-1-GFP on the cytoplasmic face of ER, we used another construct of Derlin-1 containing a Myc-tag at the C-terminus (Derlin-1-Myc) and GFP-tagged PNGase (PNGase-GFP). Identical results were obtained using these constructs (data not shown), which indicated that the epitope tag did not interfere with immunoprecipitation analysis. Non-specific interaction due to epitope tagging was ruled out as GFP -reactive bands were not observed from samples co-expressing pcDNA3.1 vector alone and Derlin-GFP (Figure 3.1A, lane 2). To confirm that PNGase and Derlin-1 interact within the cells before lysis and not during the coimmunoprecipitation procedure, PNGase-HA and Derlin-1-GFP were separately expressed in HeLa cells. Cells expressing PNGase-HA were lysed and mixed with a lysate from cells expressing Derlin-1-GFP. The mixed lysate was subjected to immunoprecipitation, overnight, with either mono-HA or mono-GFP antibodies using the same conditions as described above. Immunoblots with polyclonal anti-HA and anti-GFP antibodies demonstrated that PNGase-HA and Derlin-1-GFP did not coimmunoprecipitate when both the proteins were expressed separately and mixed together after cell lysis (data not shown). Thus, post-lysis interactions between PNGase-HA and Derlin-1-GFP did not account for the co-immunoprecipitation results.

To further rule out the possibility of a non-specific interaction between PNGase and an ER membrane protein, we tested for interaction between PNGase and the ER membrane protein calnexin. PNGase (Figure 3.1B, lane 2 upper panel) was unable to coimmunoprecipitate calnexin (Figure3.1B, lane2, lower panel). Figure 3.1B shows a

negative control expressing pCDNA3.1 vector alone. Together, these data suggest that Derlin-1 specifically interacts with PNGase and forms a complex with it in close proximity to the ER.

2. Derlin-1 interacts with PNGase *via* its cytosolic domain at the C-terminus: As previously mentioned, both the N and C-termini of Derlin-1 are in the cytosol [40]. We therefore expected that the association of PNGase would occur either through the cytosolic N- or C-terminus of Derlin-1. To determine the domain of Derlin-1 responsible for mediating interaction with PNGase a variety of Derlin-1 truncation constructs were prepared. The expression and localization of these was studied by immunofluorescence microscopy (data not shown). It was observed that all constructs except Derlin-1-GFPΔ187-251 were mislocalized in HeLa cells. Derlin-1-GFPΔ187-251 showed perinuclear localization, similar to the wild type. However no co-localization was observed with PNGase-HA, suggesting that cytosolic C-terminus of Derlin-1 is required to bring PNGase in close proximity to the ER. Upon Western blot analysis we found that Derlin-1-GFPΔ187-251 was expressed to the same level as full-length Derlin-1 (Figure 3.2B, lane 4, right panel), while the expression of the other constructs could not be detected (data not shown). We therefore used this construct for our subsequent studies (Figure 3.2A).

HeLa cells co-transfected with the pcDNA 3.1 vector alone and Derlin-1-GFP (Figure 3.2B, lane 1,), pEGFP and PNGase-HA (Figure 3.2, lane 2), PNGase-HA and Derlin-1-GFP (Figure 3.2B, lane 3) or PNGase-HA and Derlin-1-Δ187-251GFP (Figure 3.2B, lane 4) were lysed in digitonin buffer and subjected to immunoprecipitation with polyclonal anti-GFP antibody. The immune complex was recovered on Protein A/G agarose beads, following elution with SDS-PAGE sample buffer, and then analyzed by SDS/PAGE.

Immunoblotting revealed the co-precipitation of full-length PNGase-HA (Figure 3.2B, lane 3, lower panel). The pEGFP vector alone did not co-precipitated PNG1-HA (Figure 3.2B, lane 2, lower panel). No co-precipitation of PNG1-HA was observed with Derlin-Δ187-251GFP Figure 3.2B, lane 4, lower panel). This result provides evidence that the C-terminus of Derlin-1 is essential for interaction with PNGase.

Localization of PNGase and Derlin-1: An earlier study examining the distribution of endogenous PNGase observed extensive co-localization of PNGase with calnexin, a well-known ER marker [104]. Overexpressed full length PNGase-HA and Derlin-1-GFP were found to be co-localized with each other around the ER (Figure 3.3A), although the presence of cytoplasmic PNGase was also observed. The localization pattern of overexpressed PNGase in this study is therefore consistent to that observed for the endogenous protein [104]. As shown in Figure 3.3 (panels B and C) overexpressed PNGase-HA and Derlin-1-GFP were also found to co-localize with the ER membrane protein, calnexin, included as an ER marker. Lilley et al. have previously demonstrated the co-localization of Derlin-1 with calnexin [40]. The pEGFP vector alone (control) did not show any co-localization with PNGase (Figure 3.3D).

4. A Small fraction of PNGase associates with the ER: We examined the distribution of PNGase-HA and Derlin-1-GFP in HeLa cells by subcellular fractionation. Subcellular fractionation was performed on 5 x 10^7 cells as described in experimental procedures. The same volume of digitonin buffer was used for each fraction in order to compare the distribution of PNGase and Derlin-1 in the membrane-free cytosol and the ER membrane fractions. The total cell lysate obtained from 5 x 10^7 cells was used to estimate the total protein amount. All fractions, namely, the total cell lysate, membrane free cytosol or ER

membrane fraction, were immunoprecipitated with anti-HA or anti-GFP followed by SDS/PAGE and immunoblotting. The same fractions were analyzed to detect calnexin as a marker for the ER and α-tubulin as a marker for cytosol. The results in Figure 3.4A, lane 1 shows that Derlin-1 (bottom left panel) and PNGase (top left panel) along with the subcellular markers calnexin (top left panel) and tubulin (bottom left panel) were present in the total cell lysate, as expected. The bulk of PNGase was found in the membrane-free cytosolic fraction (Figure 3.4A, top left panel, lane 2); much less PNGase was detected in the membrane fraction (Figure 3.4A, top left panel, lane 3). In contrast, almost all of the Derlin-1 was present in the membrane fraction (Figure 3.4A, bottom left panel, lane 3) with virtually none in the membrane-free cytosol (Figure 3.4A, bottom left panel, lane 2). Calnexin showed the same distribution as Derlin-1, with all of it in the ER membrane fraction (Figure 3.4A, top right panel, lane 3) and none in the cytosolic fraction (Figure 3.4A, top right panel, lane 2). Tubulin as a marker for cytosol was also analyzed in all three fractions as shown in Figure 3.4A, bottom right panel. As expected, no tubulin was detected in the ER membrane fraction, excluding the possibility of cytosolic contamination.

Since we immunologically established the presence of PNGase in the membrane-free cytosol and ER membrane fraction, we then determined the level of enzyme activity of PNGase in both fractions. As shown in Figure 3.4B PNGase activity (using [^{14}C] labeled glycopeptide as a substrate) was detected in total cell lysate, membrane free cytosol and ER membrane fraction. Maximum activity was found in the total cell lysate (Figure 3.4B, lane 2) followed by the cytosolic fraction (Figure 3.4B, lane 3) while the lowest level of activity was detected in ER membrane fraction (Figure 3.4B, lane 4). As observed in lane 1, no PNGase activity was detected in the cell extract expressing pcDNA3.1 vector alone. Endogenous PNGase activity could not be detected in cells expressing pcDNA 3.1 vector alone under the

experimental conditions used because only 10µl out of 1.0 ml was used in the assay. Additionally, no PNGase activity was detected in a mutant PNGase where a catalytic cysteine is mutated to alanine [104], included as a negative control. Thus the data obtained from subcellular fractionation and PNGase activity assay were found to be consistent.

5. The N-terminus of PNGase interacts with Derlin-1: To determine the domain of PNGase interacting with Derlin-1 we evaluated a collection of PNGase constructs containing different domains with GFP-tag at the C-terminus as shown in Figure 3.5A. The N-terminus contains a highly conserved PUB (Peptide:N-glycanase/UBA or UBX-containing protein domains) [108] or PUG (Peptide:N-glycanase and other putative nuclear UBA or UBX domains) [132] domain implicated in protein-protein interactions. The C-terminal extension of PNGase in higher eukaryotes binds N-glycans. As shown in Figure 3.5A six constructs of PNGase with different domain boundaries, N-terminus alone (ΔMC), N-terminus and M-domain (ΔC), M-domain alone (ΔNC), M-domain and C-termini (ΔN) as well as C-terminus alone (ΔNM), were prepared so as to determine which domain of PNGase was capable of interacting with Derlin-1.

The constructs were co-expressed with Derlin-1-Myc in HeLa cells. Cells were lysed in digitonin buffer and co-immunoprecipitation in the total cell extract was performed with monoclonal anti-Myc antibody as well as reverse co-immunoprecipitation with polyclonal anti-GFP antibody. Samples were subjected to SDS/PAGE followed by immunoblotting. As shown in Figure 3.5B, HeLa cells co-expressing pcDNA4.0 vector and PNGase-GFP showed no interaction (Figure 3.5B, lane 1), which served as a negative control. This was further confirmed when no co-precipitation was observed by co-expression of pEGFP vector alone and Derlin-1-Myc (Figure 3.5B, lane 2). As expected using anti-

Myc-antibody, full length PNGase-GFP co-immunoprecipitated with Derlin-1-Myc (Figure 3.5B, lane 3). Given that PNGase has a PUB/PUG domain at the N-terminus we hypothesized that it might be responsible for the interaction with Derlin-1. This was indeed the case, as the N-terminus containing ΔMCPNGase-GFP co-precipitated with Derlin-1-Myc in both immunoprecipitation with anti-Myc antibody (Figure 3.5B, lane 4). The PNGase-GFP construct with both the N and M-domains also was found to interact with Derlin-1-Myc (Figure 3.5B, Lane 5). In contrast, PNGase-GFP constructs lacking the N-terminal segments (ΔNC, ΔN and ΔNM) failed to interact with Derlin-1-Myc (Figure 3.5B, lane 6, 7 and 8).

6. *In vitro* GST-binding assay and gel filtration confirm the interaction of the C-terminal domain of Derlin-1 with the N-terminal domain of PNGase: Although the interaction of PNGase with Derlin-1 in total cell lysate of HeLa cells was analyzed in co-immunoprecipitation experiments, these results do not exclude the possibility of an indirect interaction of PNGase with Derlin-1 *via* another partner. To test for direct interaction between PNGase and Derlin-1 we used a GST-binding assay. A fusion protein containing GST and the cytosolic domain of Derlin-1 (GST-Derlin-1-C) was bound on glutathione beads. Purified recombinant full length PNGase-His (PNGase-His) or PNGase-His with the N-terminus alone (ΔMCPNGase-His) were incubated with GST-Derlin-1-C bound on GSH agarose beads. Purified PNGase-His and ΔMCPNGase-His are shown in Figure 3.6, lanes 1 and 4 (top panel). PNGase-His and ΔMCPNGase-His did not bind to GST alone (Figure 3.6, lanes 2 and 5) whereas PNGase-His and ΔMCPNGase-His bound to GST-Derlin-1-C (Figure 3.6, lanes 3 and 6). Thus, the *in vitro* GST-binding assay confirms the N-terminus of PNGase indeed binds directly to the cytosolic C-terminus of Derlin-1.

Discussion

As discussed previously, PNGase has been reported to be localized in the cytosol [125], and also reported to be associated with the ER membrane [104,133]. Conflicting evidence also suggests localization in the ER lumen [134]. PNGase in higher eukaryotes acts on a diverse range of misfolded glycoprotein substrates, namely immunoglobulin subunits [135], HCs [136], a ribophorin I variant [137], cog thyroglobulin mutant [138] cystic fibrosis transmembrane regulator [139] and T-cell receptor (TCR) α-subunits [140]. In a study using a novel PNGase inhibitor (Z-VAD(OMe)-fmk) [141] it was found that two well characterized substrates of PNGase, TCRα and HCs, showed different cellular sites of deglycosylation. In the absence of the PNGase inhibitor the deglycosylated form of HCs were observed in the cytosol as well as in the ER membrane fractions. In contrast, all HCs remained glycosylated in the presence of PNGase inhibitor, regardless of their subcellular distribution. This indicates that the process of dislocation of HCs is not dependent upon deglycosylation. The finding that deglycosylated HCs remain associated with the ER membrane fraction in the absence of PNGase inhibitor, suggests a mechanism in which HCs encounter PNGase at the ER membrane. During HC dislocation PNGase removes N-linked glycans prior to the release of the polypeptides into the cytosol for subsequent degradation by the proteasome. In fact, in the studies of Wiertz *et.al* only the deglycosylated forms of HCs were observed to be associated with the Sec61 complex of the ER membrane, indicating that glycoproteins in this channel must also encounter N-glycanase [36]. Another example of the ER membrane-localized deglycosylation is the protein ovalbumin in which site specific N-deglycosylation was attributed to an ER situated N-glycanase [133]. In contrast to HCs, TCRα apparently utilizes a different dislocation machinery for degradation [141]. Fully

glycosylated TCRα were found in the membrane fraction in the presence of proteasome inhibitors suggesting that TCRα dislocation is coupled to degradation.

In a prior report, it was found that endogenous PNGase could be detected associated with the ER in a variety of cell lines including HeLa [104]. In the current study we have carried out overexpression of epitope-labeled PNGase, and confirmed the earlier observations as well as identified the protein that mediates PNGase binding to the ER. The fact that MHC class I HCs are PNGase substrates and that deglycosylated HCs are detected in the ER membrane associated with Derlin-1 [40] prompted us to explore the possible interaction between Derlin-1 and PNGase. Initially we carried out immunoprecipitation experiments to detect interaction between Derlin-1 and PNGase. Derlin-1 and PNGase were found to co-immunoprecipitate regardless of the epitope tag. Derlin-1 contains four transmembrane domains and the N and C-termini are located in the cytosol [40,41]. Therefore, we predicted that the observed interaction of PNGase with Derlin-1 could occur via Derlin-1's N or C-terminus. Co-immunoprecipitation with the deletion constructs of Derlin-1 revealed that the Derlin-1 lacking its C-terminus cytosolic domain failed to co-precipitate with PNGase. Next we showed that the cytosolic C-terminus of Derlin-1 interacted directly with PNGase. Several experimental approaches established that it was the N-terminus of PNGase that bound to Derlin-1. The most conserved region of PNGase, the M-domain which contains the active site residues [105] does not interact with Derlin-1, suggesting that extension of N-terminal PNGase in higher eukaryotes (which is absent in yeast PNGase), may facilitate deglycosylation of misfolded glycoprotein substrates by association of the deglycosylating enzyme with the ER membrane. Thus, it seems likely that the cytosolic C-terminus of Derlin-1 interacts with the N-terminus of PNGase and thus brings the deglycosylating enzyme in close proximity to the site where misfolded

glycoproteins exit the ER. Consistent with this idea are the findings that 1) the total cell lysate of HeLa cells when subjected to gel filtration (data not shown) co-eluted of Derlin-1 with a small fraction of PNGase and 2) subcellular fractionation studies confirmed this observation. Despite the low abundance of PNGase at the ER membrane, its level is sufficient to co-precipitate almost all of the Derlin-1 (data not shown).

Based on all of our results we propose the model shown in Fig. 7, in which PNGase is present in the cytosol as well as associated with the ER membrane. The HCs bind transiently to Derlin-1 and are dislocated across the ER into the cytosol. It has previously been shown that deglycosylated glycoprotein bind to HR23B [104]. As illustrated in this study, PNGase bound to the ER membrane interacts with the C-terminus of Derlin-1 *via* its N-terminus. The N-terminus of PNGase has already been shown to mediate the binding of HR23B [104], which in turn interacts with S_4 subunit at the base of the proteasome lid. Since the majority of PNGase is in the cytosol, interaction of PNGase with Derlin-1 at the ER membrane may be required for the deglycosylation of a subset of misfolded glycoprotein substrates, such as the HCs. Glycoproteins like TCRα, on the other hand, may be deglycosylated by the free cytosolic enzyme. As shown in figure 7, a different subset of misfolded proteins perhaps utilize the retrotranslocation channel formed by Sec61 complex which interacts with the 19S particle of the proteasome [38]. This interaction suggests that degradation of misfolded proteins indeed takes place in close vicinity of the ER membrane.

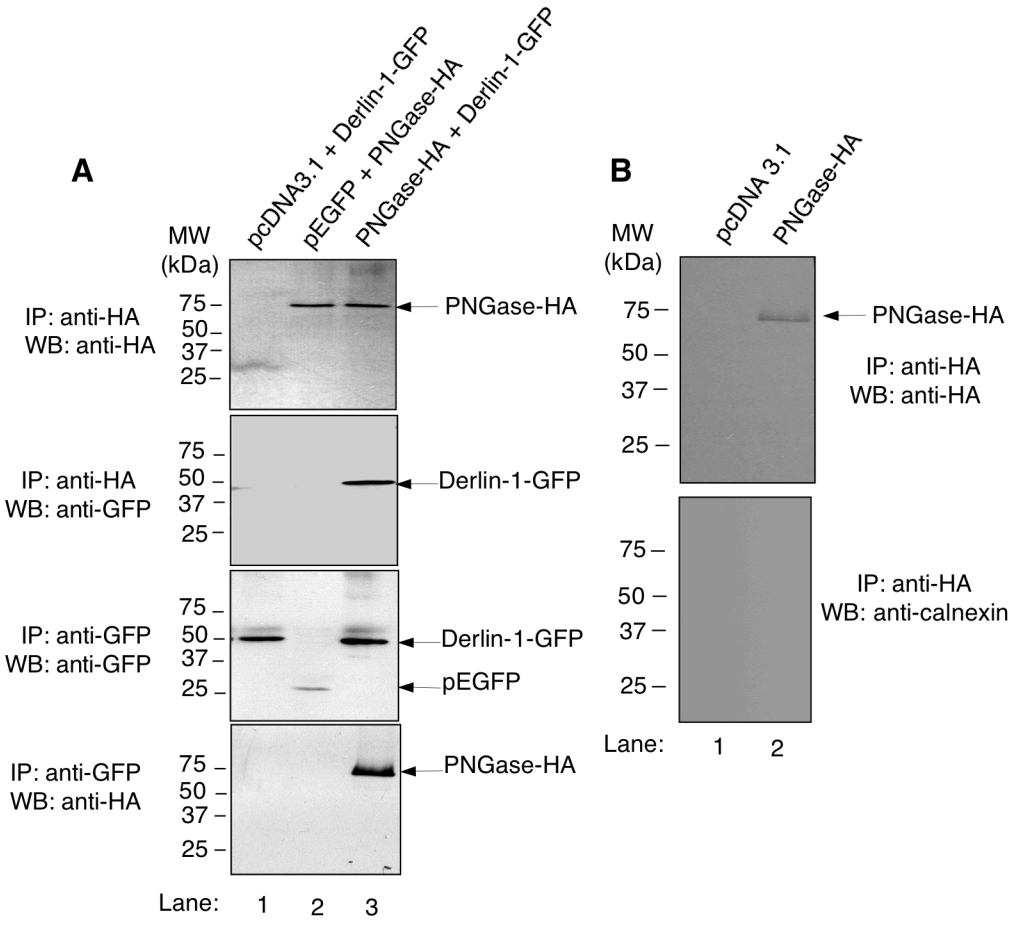

Figure 3.1 PNGase interacts with Derlin-1 *in vivo*. A. Interaction of PNGase with Derlin-1 analyzed by co-immunoprecipitation. HeLa cells were co-transfected with pcDNA3.1 vector and Derlin-1-GFP (lane 1), pEGFP and PNGase-HA (lane 2) or PNGase-HA and Derlin-1-GFP (lane 3) and immunoprecipitated with monoclonal anti-HA antibody. Reverse immunoprecipitation was also performed with polyclonal anti-GFP antibody. Immune complexes obtained were resolved on SDS-PAGE and immunoblotted with antibodies as indicated B. Calnexin, an ER membrane protein, did not co-precipitate with PNGase. HeLa cells transfected with pcDNA3.1 vector alone (lane 1) and PNGase-HA (lane 2) were immunoprecipitated with monoclonal anti-HA antibody, resolved on SDS-PAGE and immunoblotted with polyclonal anti-HA and polyclonal anti-calnexin antibodies as indicated.

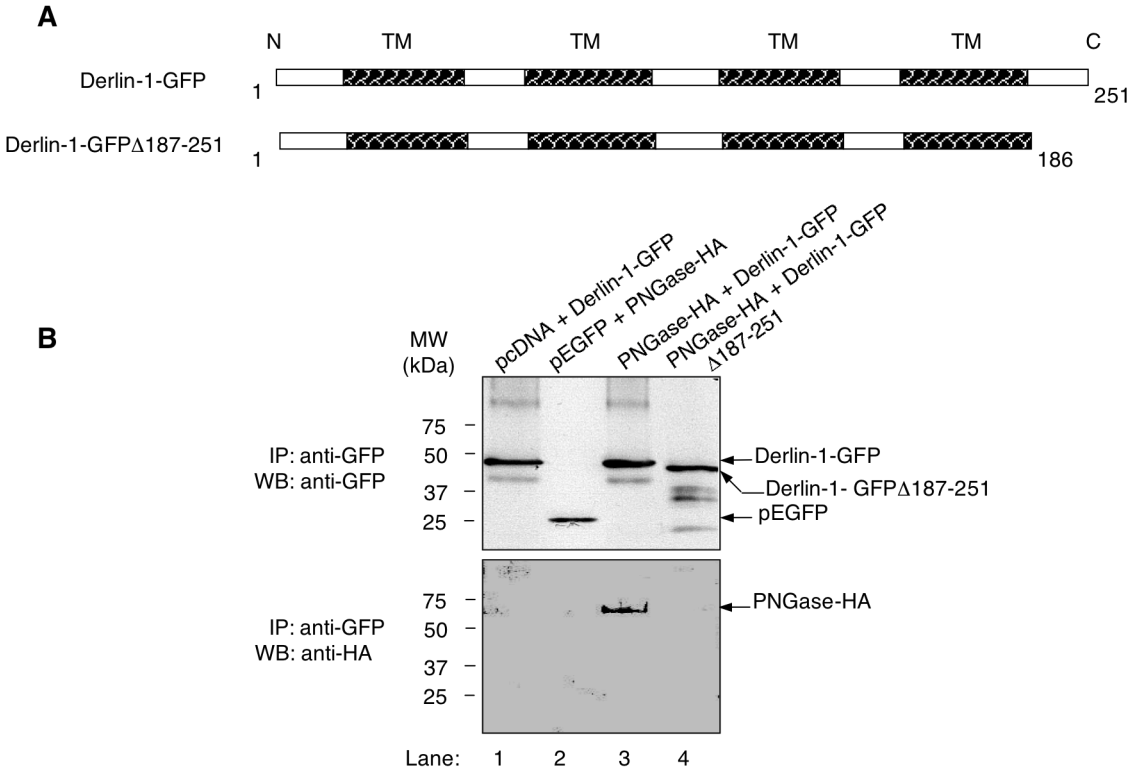

Figure 3.2. Cytosolic C-terminus of Derlin-1 interacts with PNGase. A. Constructs of Derlin-1-GFP used in this study. B. Co-immunoprecipitation of PNGase-HA with Derlin-1-GFP: HeLa cell lysate co-expressing pcDNA3.1 vector and Derlin-1-GFP (lane 1), pEGFP vector and PNGase-HA (lane 2), PNGase-HA and Derlin-1-GFP (lane 3) or PNGase-HA and Derlin-1-GFPΔ187-251 (lane 4) were immunoprecipitated with monoclonal anti-GFP antibody (left panel). Samples obtained after immunoprecipitation were resolved on SDS-PAGE and analyzed by Western Blotting with polyclonal anti-GFP antibody (top panel) or anti-HA antibody (bottom panel).

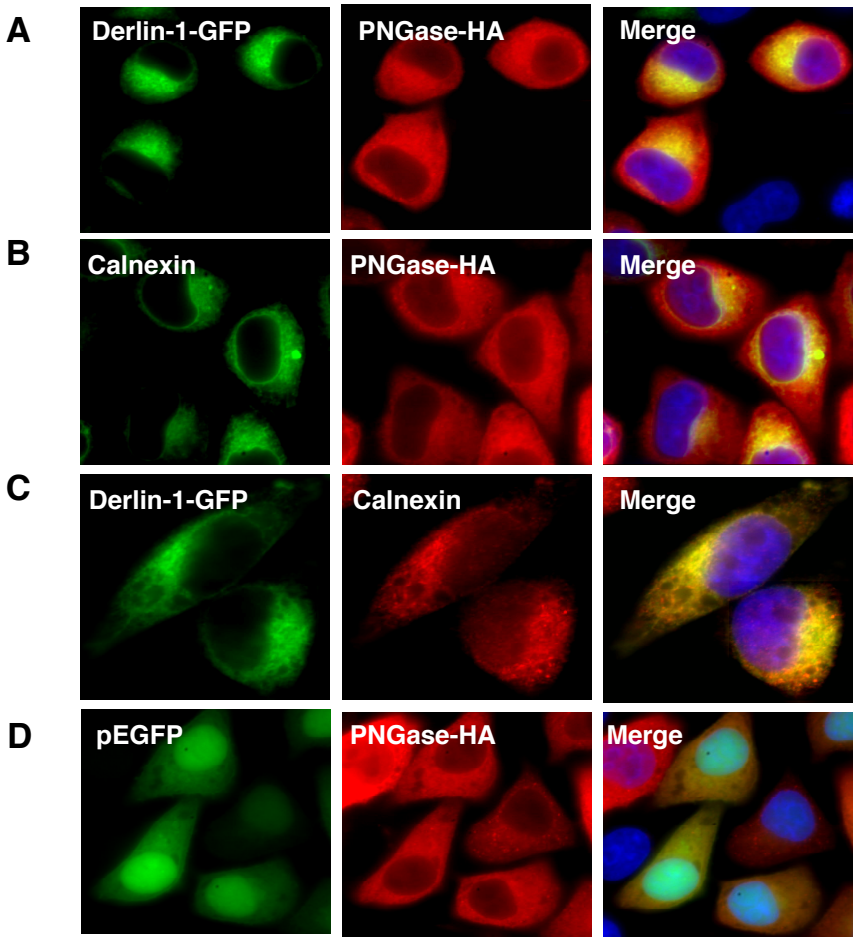

Figure 3.3 PNGase co-localizes with Derlin-1. HeLa cells were cotransfected with Derlin-1-GFP and PNGase-HA (A) or pEGFP and PNGase-HA (D) or tranfected with PNGase-HA or Derlin-1-GFP alone (B and C respectively). A. PNGase-HA (red) was found to colocalize with Derlin-1-GFP (green), as shown in the merged image (right panel, yellow shows colocalization). B. PNGase-HA (red) was also shown to colocalize with another ER membrane protein, calnexin (green), as seen in the merged image (right panel). C. Derlin-1-GFP (green) and calnexin (red) colocalize (merged image, right panel) in the ER. D. GFP protein, when expressed alone (green), does not colocalize with PNGase-HA (red), as seen in the merged image (right panel). DAPI (blue) shows nuclear staining.

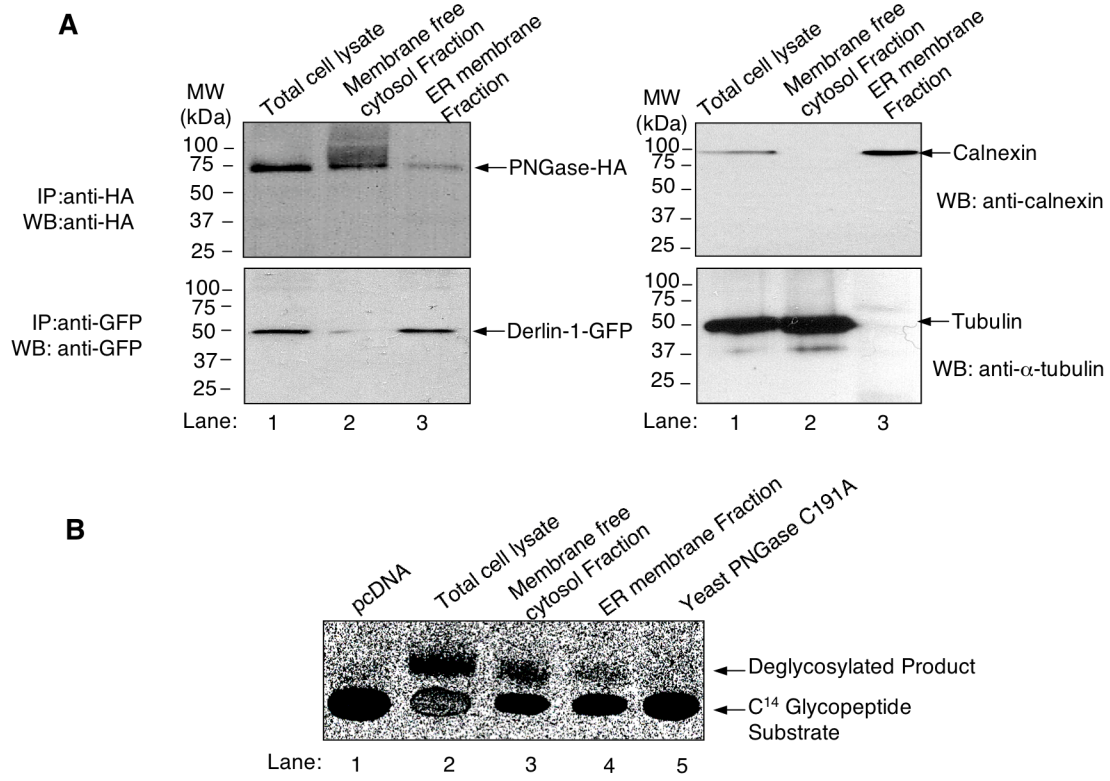

Figure 3.4 Small fraction of membrane bound PNGase associates with ER.
A. Distribution of PNGase in HeLa cells. Subcellular fractionation was performed as described in Material and methods. Shown are HeLa cell lysate (lane 1), membrane free cytosolic fraction (lane 2) and ER membrane fraction (lane 3). Equal volumes of all the fractions were subjected to immunoprecipitation with monoclonal anti-HA (for PNGase-HA) and polyclonal anti-GFP (for Derlin-1-GFP) followed by SDS/PAGE and immunoblotting with antibodies as indicated. Anti-calnexin and anti-α-tubulin antibodies were used as markers for ER and cytosol respectively (top and bottom right panels respectively). C. PNGase enzyme activity in subcellular fractions. Activity assay for PNGase was performed using 10μl of each fraction. Shown are extracts from HeLa ells expressing pcDNA3.1 vector alone (lane 1), or HeLa extracts overexpressing PNGase-HA (lane 2), membrane free cytosolic fraction (lane 3), ER membrane fraction (lane 4) and lysate from *E. coli* cells expressing yeast PNGase mutant C191A (lane 5) used as a negative control.

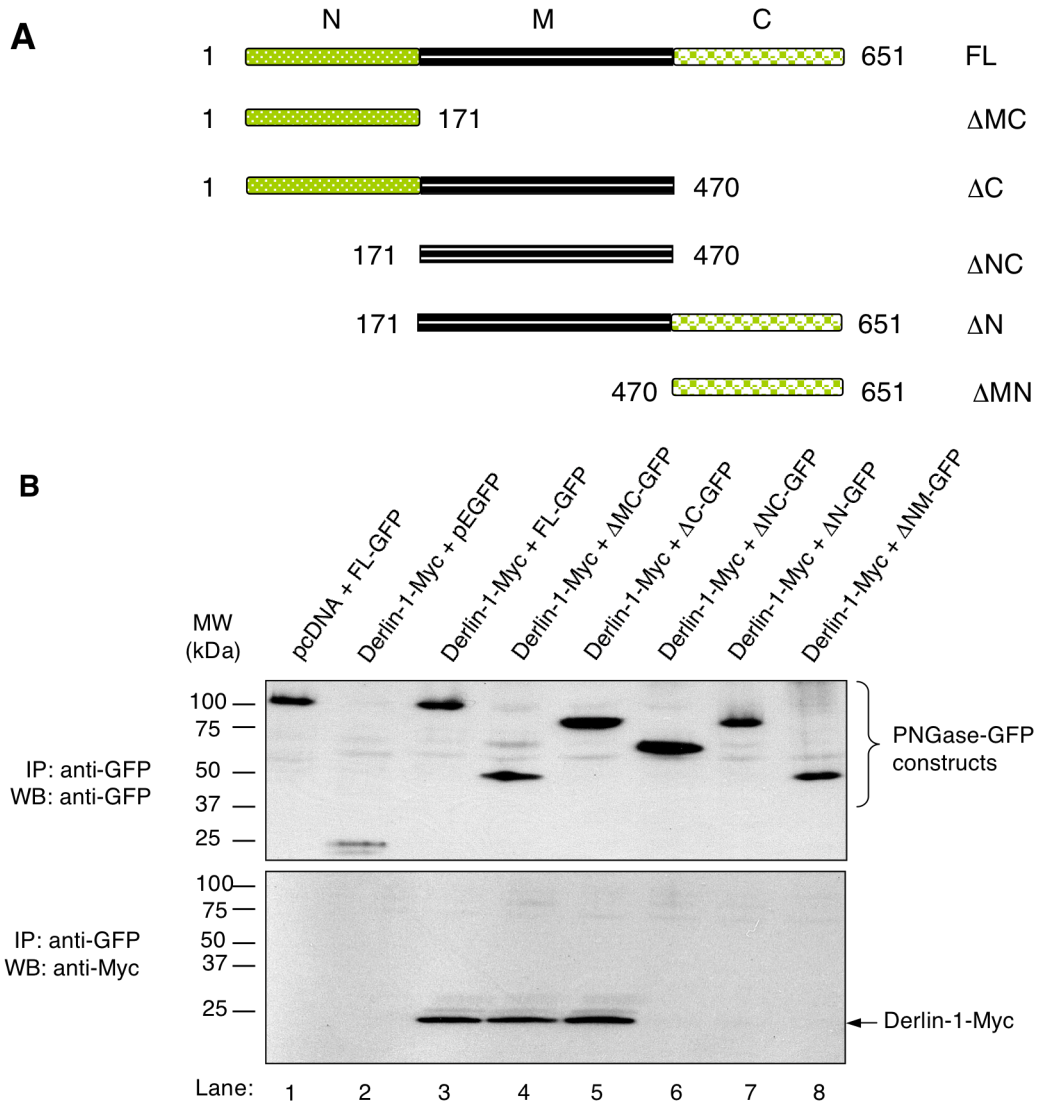

Figure 3.5 The N-terminus of PNGase interacts with Derlin-1. A. Deletion constructs of PNGase used in this study. B. HeLa cell lysates obtained from cells co-expressing pcDNA3.1 vector alone and PNGase-GFP (lane 1), pEGFP vector alone and Derlin-1-Myc (lane 2), Derlin-1-Myc and PNGase-GFP (lane 3), Derlin-1-Myc and ΔMCPNGase-GFP (lane 4), Derlin-1-Myc and ΔCPNGase-GFP (lane 5), Derlin-1-Myc and ΔNCPNGase-GFP (lane 6), Derlin-1-Myc and ΔNPNGase-GFP (lane 7) and Derlin-1-Myc and ΔNMPNGase-GFP (lane 8) were subjected to immunoprecipitation with monoclonal anti-Myc antibody. Immune complexes bound on protein A/G agarose beads were eluted with 2 x SDS-PAGE sample buffer, resolved on SDS-PAGE and analyzed by western Blotting with polyclonal anti-GFP antibody (upper panel) and polyclonal anti-Myc antibody (lower panel).

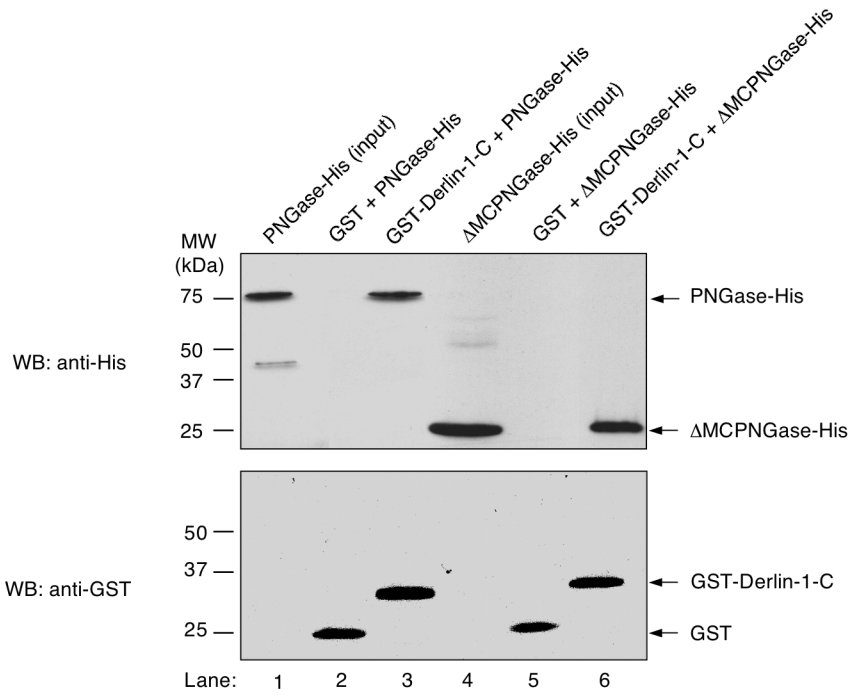

Figure 3.6 Interaction of N-terminus of PNGase with the cytosolic C-terminus of Derlin-1 was confirmed by *in vitro* GST-binding assay. GST or GST-Derlin-1-C expressed in *E. coli* DH5α were purified and allowed to bind on GSH-agarose beads. *E. coli* BL21(DE3) cell lysate expressing full length PNGase-$(His)_6$ and ΔMCPNGase-$(His)_6$ were used for *in vitro* GST-binding assay followed by SDS-PAGE and immunoblotting with anti-His and anti-GST antibodies (upper and lower panels respectively. Shown are PNGase-$(His)_6$ in cell lysate (lane 1), GSH beads incubated with GST alone and PNGase-$(His)_6$ (lane 2), GSH beads carrying GST-Derlin-1-C and PNGase-$(His)_6$ (lane 3), cell lysate ΔMCPNGase-$(His)_6$ (lane 4), GSH beads incubated with GST alone and ΔMCPNGase-$(His)_6$ (lane 5), GSH beads incubated with GST-Derlin-1-C and ΔMCPNGase-$(His)_6$ (lane 6).

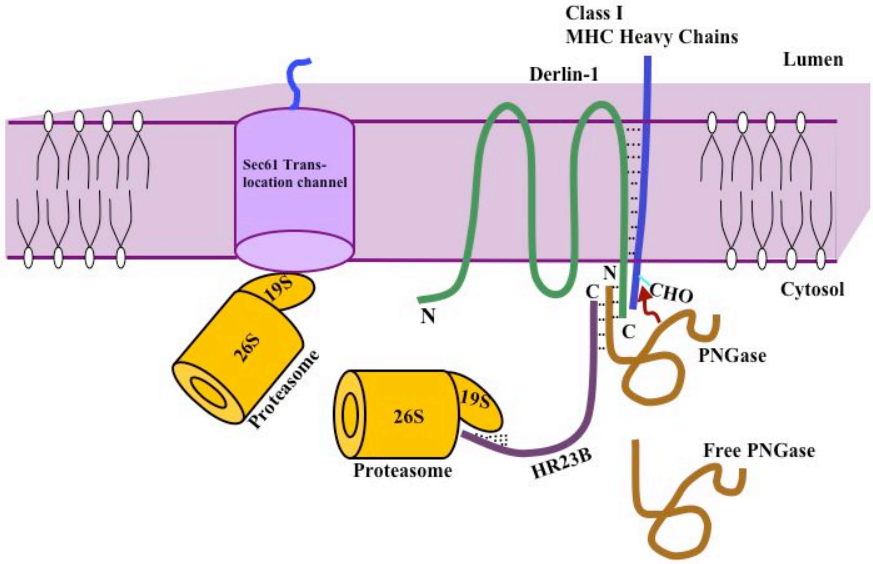

Figure 3.7 Model of PNGase mediated deglycosylation of misfolded glycoproteins retrotranslocated *via* channel formed by Derlin-1 (HCs have been used as model substrates; there may be other glycoprotein substrates that utilize this pathway). Derlin-1 interacts with the HCs and targets these to the cytosol for degradation by the proteasome. As shown, the N-terminus of PNGase interacts with the cytosolic C-terminus of Derlin-1 and consequently facilitates deglycosylation of the HCs. CHO indicates the carbohydrate chains. Upon deglycosylation, HR23B acts as a receptor for deglycosylated (polyubiquitinated) substrates [104] and targets them to the proteasome for degradation through its interaction with the S_4 proteasomal subunit. (Other Derlin-1 interacting partners p97 and VIMP [41] are not shown in this model). Retrotranslocation channel formed by Sec61 complex is also shown in this model that may be involved in the translocation of a different subset of misfolded protein substrates. Recently, the Sec61 channel has been demonstrated to be the membrane receptor for proteasome, suggesting that the degradation of misfolded proteins is coupled to their dislocation. Whether the retrotranslocation channel formed by Derlin-1 also directly interacts with the proteasome remains to be determined.

Chapter 4. Gp78 localizes exclusively to the ER

Summary

Gp78 has previously been established as an ER resident E3-ligase involved in the turnover of misfolded glycoproteins such as CD3-δ. However, in the past it has been purported that gp78 is identical to the autocrine motility factor receptor (AMFR), which resides in specialized microdomains (caveolae) of the cell surface. At the cell surface, AMFR has been reported to bind its ligand autocrine motility factor (AMF), and subsequently induce the internalization of this complex into the ER. This interaction between AMF and AMFR has been proposed to occur via the N-linked glycan modification of AMFR.

We undertook a study to evaluate the topology and the subcellular localization of gp78. Using the methodology of confocal microscopy and cell-surface biotinylation assay, we demonstrate that gp78 localizes exclusively to the ER. Trypsin protection assay of microsomes indicates that both the N and C-termini of gp78 reside in the cytsol. Finally, our assessment of the gp78 N-glycosylation status has revealed that this protein is not modified by N-linked glycans, as previously proposed.

Materials and Methods

1. Plasmid Constructs: cDNA corresponding to full-length mouse gp78 (1-643 amino acid residues) was amplified by PCR and cloned into pcDNA4.0 (Invitrogen, Carlsbad, CA) using *5'* EcoRV, *3'*XbaI to obtain a C-terminal Myc-tagged gp78 construct. Similarly, *5'*EcoRI and *3'* BamHI sites were used to clone the full length mouse gp78 cDNA into pCMVTag3C (Clonetech, Palo Alto, CA) to generate a N-terminal Myc-tagged gp78 construct. Pancreatic protein disulfide isomerase (PDIp) cDNA was amplified by PCR and cloned into pEGFP-N1 vector (Clonetech, Palo Alto, CA). HA-tagged caveolin-1 construct was a gift from Dr. Deborah Brown of Department of Biochemistry and Cell Biology, Stony Brook University.

2. Cell Culture and Transfection protocols have been described in Chapter 3.

3. Antibodies and Reagents: The 3F3A anti-gp78 rat monoclonal antibody was a gift from Dr. Avraham Raz (Wayne State University). Mouse monoclonal, rabbit polyclonal Myc antibodies and rabbit polyclonal β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal HA antibody was obtained from Covance. Calreticulin antibody (rabbit polyclonal) and Sec61-β antibody (rabbit polyclonal) were purchased from Affinity Bioreagents (Golden, CO), and Upstate (Millipore, Temecula, CA) respectively. Mouse antibody to PAN-Cadherin was obtained from Sigma (St. Louis, MO). AlexaFluor-555 (AF-555), 4', 6-diamidino-2-phenylindole (DAPI) and fluorescien isothiocyanate (FITC) were purchased from Molecular Probes (Invitrogen detection technologies, Eugene, OR). EZ-Link™ NHS-Biotin and

ImmunoPure immobilized streptavidin was purchased from Pierce (Rockford, IL). Complete™ protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). The bacterial deglycosylating enzyme, endoglycosidase H (Endo H) was purchased from New England Biolabs (Ipswich, MA).

4. Immunoflourescence and Microscopy: HeLa cells were cultured on coverslips in 6 well dishes and transfected with plasmids as described above. Twenty-four hours post-transfection, the cells were fixed in 3% paraformaldehyde solution for 20 min on ice and washed briefly for three times with phosphate buffered saline (PBS). Cell-permeabilization was performed in 0.5% TritronX-100 (in 1X PBS) for 5 min on ice. The cells were washed and incubated in blocking buffer (3% bovine serum albumin, 10mM glycine in 1X PBS) for 2hr at room temperature and were subsequently incubated with primary antibodies as indicated (anti-Myc, anti-HA, 1:200 dilution, anti-calreticulin 1:100 dilution respectively). Goat anti-rabbit or anti-mouse conjugated to FITC were used in 1:250 dilution and goat anti-rabbit or anti-mouse conjugated to AF-555 were used in a dilution of 1:1000. For nuclear DNA staining, DAPI was used. Confocal-microscopy was performed using a Zeiss Axiovert confocal microscope (Zeiss, Thornwood, NY) followed by deconvolution analysis of the Z-stacks.

5. Cell Surface Biotinylation: HeLa cells were cultured in 6-well dishes and transiently transfected with gp78-pcDNA4.0 plasmid. Twenty-four hours post-transfection, the cells were washed three times in ice-cold Hank's Buffered Salt Solution (HBSS) and treated with 1ml of 2.5mM EZ-Link™ NHS-Biotin solution prepared in HBSS. For mock-biotinylation, the cells were simply treated with HBSS containing

dimethoxy sulfoxide (DMSO, used for preparing stock solutions of NHS-biotin). The cells were incubated on ice for 10 min and the biotinylation mixture was aspirated and the process repeated for one more time. The reaction was stopped by the addition of 2ml ice-cold 100mM Tris.Cl pH7.4 (prepared in HBSS) to each dish. After aspiration of 100mM Tris.Cl, the process was repeated once and the reaction mixture was allowed to incubate for 15 min on ice, with a subsequent wash with HBSS. Cell lysis was carried out by the addition of 500µl RIPA buffer (50mM Tris.Cl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) containing 5mM EDTA and protease inhibitor cocktail. The dishes were incubated at 4ºC for 15min on a rocker. The lysate was centrifuged at 14,000 rpm for 10 min. To 500µl supernatant obtained by this centrifugation, 30µl of ImmunoPure immobilized streptavidin was added. The tubes were incubated at 4ºC overnight. The streptavidin beads were harvested by centrifugation and washed three times with RIPA buffer and treated with 2X SDS-PAGE sample-buffer and boiled for 5 min. The samples were resolved by SDS-PAGE followed by Western Blot analysis using Myc, β-actin and cadherin antibodies.

6. Trypsin Protection Assay: HeLa cells were cultured in 100 mm dishes and transiently transfected with gp78-pcDNA4.0 or gp78-pCMVTag3C plasmids. Forty-eight hours post-transfection, the cells were scraped and collected in 450µl of sucrose buffer (50mMTris.Cl pH 8.0, 0.32M sucrose, 1mM EDTA, 1mM DTT). The cell suspension was passed through a tissue homogenizer several times, while incubating on ice. The lysate was centrifuged in cold at 4000 rpm for 5 min. The supernatant was divided into four 100µl aliquots, and treated with 0, 5, 15 or 20µg of trypsin respectively. The tubes were incubated at 4ºC for 15 min on a rocker. The reaction was

stopped by the addition of 100μl of 2X sample buffer, with subsequent boiling for 5 min. The samples were then resolved on 10% SDS-PAGE and analyzed by Western Blotting with appropriate antibodies.

7. Deglycosylation assay: HeLa cells cultured in 35mm dishes were transiently transfected with gp78-pcDNA4.0 and PDIp-pEGFP-N1 respectively or left untransfected. Twenty-four hours post-transfection, the cells were washed three times with 1X PBS and harvested in 70μl of 2xSDS-sample buffer. Each sample was boiled for 10min and divided equally into two tubes. One tube was treated with Endoglycosidase H as per manufacturer's protocol, while the other was left untreated. Subsequently, all the samples were analyzed by SDS-PAGE and Western blotting, using the appropriate antibodies.

Results

1. Gp78 localizes exclusively to the ER: Several prior studies on gp78 (AMFR), have shown that the protein localizes to the ER as well as the plasma membrane [81,82]. In fact, it has been proposed that gp78 acts as a receptor for autocrine motility factor or AMF and binds AMF at the cell-surface. The ligand-receptor complex is subsequently internalized to a "smooth ER tubule" [81].

To study the localization pattern of gp78 in HeLa cells, confocal microscopy of HeLa cells transiently transfected with gp78-pcDNA4.0 was performed. HA-tagged caveolin-1 was co-transfected into the HeLa cells to serve as a marker for the cell-surface. Caveolin-1 localizes to specialized cell surface microdomains known as caveolae and is involved in endocytosis of molecules such as cholera toxin into the intracellular sub-compartments. Caveolin-1 may sometimes also be observed in the Golgi compartment and in vesicles trafficking from the Golgi to the plasma membrane. Interestingly, gp78/AMFR was previously described to be present in the caveolae and endocytose in a caveolin-1-dependent manner. Therefore, we rationalized that caveolin-1-HA would serve as an excellent marker to study the cell surface distribution of AMFR.

As seen in Figure 4.1A, C-terminally Myc-tagged gp78 when co-expressed with HA-tagged Caveolin-1, showed no co-localization with HA-tagged Caveolin-1. The magnified 3-D rendering of a small area of the cell cross-section, clearly demonstrates that gp78 and Caveolin-1 do not co-localize (Figure 4.1A). Therefore it is unlikely that gp78 present in the cell-surface caveolae.

It has previously been demonstrated that gp78 is resides in the ER. To confirm this observation, HeLa cells transfected with gp78-Myc were co-stained for the ER resident protein calreticulin and Myc (for gp78). Figure 1B (merge) demonstrates that gp78-Myc co-localizes with the ER marker calreticulin. This result is therefore consistent with the prior reports that gp78 is an ER resident protein.

To rule out the possibility that a small amount of gp78 is present at the cell-surface and cannot be detected by confocal microscopy, we performed cell surface biotinylation assay on HeLa cells transiently transfected with gp78-pcDNA4.0. As seen in Figure 4.2, (lane1, top panel), we did not observe an association of gp78-Myc with streptavidin beads following the biotinylation reaction. This indicates that gp78 was not biotinylated. As a positive control for the biotinylation reaction, we did observe the biotinylation and subsequent association of cadherin, an abundant cell surface protein, with the streptavidin beads (Figure 4.2, lane1, bottom panel). To rule out the possibility of cell-lysis during the biotinylation reaction (and subsequent biotinylation of the cytosolic proteins) we tested for biotinylation of β-actin. We did not observe any biotinylated β-actin, indicating that the cells remained intact during this procedure (Figure 4.2, lane1, middle panel). Figure 4.2, lane 2 (top, middle and bottom panels) is the expression control for gp78, β-actin and cadherin respectively from the lysate of cells obtained after the biotinylation reaction. Figure 4.2, lane 3 shows the expected negative results after a mock biotinylation reaction (in the absence of biotinylation none of the proteins are pulled down). Cadherin no longer associates with the streptavidin beads, indicating that the affinity of the streptavidin beads is only towards biotinylated material and not cadherin in general (Figure 4.2, lane3 bottom panel). Figure 4.2, lane 4

is the expression control for gp78, β-actin and cadherin respectively, from the lysate of cells obtained after the mock-biotinylation reaction.

These results therefore conclusively establish that gp78 is not a cell surface protein but is an ER resident protein.

A similar experiment under identical conditions was performed to study the localization of endogenous gp78 rather than overexpressed gp78, using the 3F3A antibody that has been previously used to study gp78's cell-surface localization by the Raz and Nabi labs [75,81]. As observed in Figure 4.2B (right panel, left lane) we failed to detect any biotinylation of endogenous gp78 with the 3F3A antibody, while significant biotinylation of cadherin, an abundant cell surface protein was observed (Figure 4.2B top left panel, left lane). Calreticulin served as the negative control (Figure 4.2B, top middle panel, left lane) indicating that the cytosolic and ER membrane proteins were not biotinylated during this procedure. As in the previous experiment, mock biotinylation procedure indicated that binding of cadherin to the streptavidin beads was mediated only via biotin (Figure 4.2B, left panel, right lane). Expression controls of cadherin, calreticulin and gp78 are shown in the bottom panels for both biotinylation and mock biotinylation reactions (Figure 4.2B, left and right lanes respectively).

Thus, based on the combined data from confocal microscopy and biotinylation assay, it seems highly unlikely that gp78 is a cell-surface receptor. Further, similar studies performed by our collaborators at A. Weissman lab at NCI (Tsai and Weissman unpublished results, personal communication) have arrived at the same conclusion.

2. The N and C-termini of gp78 are in the cytosol: To determine the orientation of the N and C-termini of gp78, we performed a trypsin protection assay. For this purpose gp78-pcDNA4.0 (C-terminal Myc-tagged gp78) and gp78-PCMVTag-3C (N-terminal Myc-tagged gp78) were transiently transfected in HeLa cells. Forty-eight hours post-transfection, the cells were harvested and then the ER membranes were prepared and treated with increasing amount of trypsin as described in the Materials and Methods section. Figure 4.3A (top panel) shows that the C-terminus of gp78-Myc was not protected and degraded by trypsin (compare lanes 2-4 with lane1). Calreticulin (Figure 4.3A, lower panel, lanes 1-4), remained intact, indicating that the ER membranes were not leaky when trypsin treatment was performed. Similarly, in Figure 4.3B (top panel) we observed that the N-terminus of Myc-gp78 was susceptible to trypsin (compare lanes2-4 with lane1), while calreticulin (Figure 4.3B, lower panel, lanes 1-4) remains intact. Thus, our results indicate that both the N and the C-terminus of gp78 are in the cytosol.

3. Gp78 does not contain high-mannose N-glycan chains: The cytoplasmic tail of gp78 contains the N-glycosylation motif NKS (residues 599-601). It has been proposed that this site is essential for the interaction of AMF with AMFR due to the reason that an AMFR mutant lacking the putative N-glycosylation site is unresponsive to stimulation by AMF [142]. Also, based on the crystal structure of AMF it was proposed that AMF interaction with AMFR is modulated through a glycan moiety.

However, upon the analysis of the putative N-glycosylation sequence, NKS, we found that this site is flanked by the Ube2g2 binding domain, G2BR, to its left (residues 579-600) and p97 binding region to its right (residues 576-643). Both p97 and Ube2g2

are cytosolic proteins [85,143]. Because N-glycosylation of proteins occurs in the ER lumen, it is highly unlikely that this NKS motif is glycosylated.

To determine if gp78 is N-glycosylated, gp78-pCDNA4.0 was transiently transfected in HeLa cells and treated with the bacterial deglycosylating enzyme Endo H. Following Endo H treatment, samples were resolved by 8% SDS-PAGE and analyzed by immunoblotting with anti-HA antibody. As seen in Figure 4.4, middle panel (lane 2) there was no shift in molecular weight corresponding to lack of N-glycosylation compared to lane1 with no Endo H treatment. As a positive control, pancreatic protein disulfide isomerase (PDIp), which is known to have N-linked glycan modification, was treated with Endo H (Figure 4.4, right panel, lane 2) and showed a substantial mobility shift compared to untreated sample (Figure 4.4, right panel, lane 1) [144]. Similar analysis of endogenous gp78 using the 3F3A antibody yielded results identical to those from the overexpressed gp78 (Figure 4.4, left panel compare lanes 1 and 2). Therefore, gp78 lacks N-linked glycosylation.

Discussion

Gp78 was originally isolated from human B16-F1 melanoma cells as a unique, 78kDa, sialylated protein that bound to peanut lectin (PNA) [75]. Antibody to gp78 (3F3A, rat monoclonal) was shown to stimulate the motility of B16-F1 melanoma cells [75]. Autocrine motility factor (AMF), which was previously identified as a cytokine, (stimulating the motility of A2058 melanoma cells) was demonstrated to compete with the 3F3A antibody for binding to gp78. Thus, gp78 was proposed to be a receptor for AMF and was therefore called autocrine motility factor receptor (AMFR). Due to its proposed function of enhancing motility of tumor cells, and its increased expression in various types of cancers such as bladder, colorectal and esophagus, AMFR was implicated in metastasis of tumor cells. Moreover, it was demonstrated that overexpression of gp78 in NIH3T3 caused their transformation. Cloning of the human and mouse gp78 cDNA revealed a protein of 643 amino acid residues, with seven predicted transmembrane domains [145]. This data along with the observation that pertusis toxin inhibits the cytokine/motility function of AMF led to the theory that gp78/AMFR is a G-protein coupled receptor (GPCR) [146].

Previous work on AMFR also suggested that this protein localized to the cell-surface microdomains (caveolae), rich in cholesterol and caveolin-1 [81]. At the cell-surface AMFR was proposed to bind to AMF and induce internalization of the AMF-AMFR complex thereby triggering cell motility. Post-internalization, the AMF-AMFR complex was indicated to reside in the smooth-subdomain of the ER. Interestingly, it was also suggested that AMF-AMFR complex recycles to the plasma membrane via a clathrin-dependent pathway and the AMF-AMFR complex was demonstrated to co-

localize in fibrillar structures containing fibronectin. Fibronectin is involved in remodeling of extracellular matrix during cell movement. Thus, it was proposed that AMF-AMFR recycling causes the enhanced motility observed during AMF stimulation [147].

As mentioned before, AMF, a 55kDa protein, was isolated and purified from serum free conditioned medium of A2058 melanoma cells as a factor responsible for stimulating motility [148]. Subsequently, results from fragmented protein microsequencing indicated that AMF was identical to the glycolytic enzyme phosphoglucoisomerase (PGI), neruoleukin (NLK, that promotes survival of spinal and sensory neurons) and maturation factor (MF, that mediates the differentiation of myeloid leukemic cells) [149]. The lack of a signal sequence on AMF suggested that this protein did not traverse the classical secretory pathway, via the ER and Golgi, but in fact was secreted by an unknown mechanism from the cytosol into the extracellular space. Analysis of culture medium indicated that AMF was secreted at a significantly higher level by tumorigenic cells, rather than their normal counterparts.

Contradictory to a prior report, the Nabi and Raz labs have shown that the enzymatic activity of PGI is not required for its AMF function [150-152]. Based on the structural analysis of human PGI in complex with the inhibitor erythrose-4-phosphate (E4P) it was concluded that the binding of E4P inhibitor to the active site inhibits the AMF/PGI cytokine function by either competing with the glycosylated receptor (AMFR) for binding or by inducing a conformational change that prevents the interaction of AMF with AMFR [153]. By creating mutations in the predicted N-glycosylation site of AMFR, the Raz group observed marked reduction in binding

between AMFR mutants and AMF, and thus concluded that glycosylation of gp78 is essential for AMF-AMFR interaction [142].

The role of gp78/AMFR in the turnover of misfolded proteins was only recently demonstrated. As mentioned in the introduction section, gp78 is a RING type E3 Ub-ligase that polyubiquitinates proteins for destruction by the proteasome. The substrates of gp78 include the CD3-δ, HMG-CoA reductase and PiZ form of α-1-antitrypsin. However, in the course of these studies it has been noted that gp78 localizes primarily to the ER. There is no direct evidence that gp78 localizes to the cell surface.

In collaboration with the A.Weissman lab at NCI, we undertook a study to clarify ambiguities regarding gp78's subcellular localization and glycosylation status. We have employed the techniques of confocal microscopy and cell surface biotinylation assay to address the issue of gp78's subcellular localization. The expression pattern of gp78 was studied in HeLa cells. This cell line, in prior reports, has been documented to produce higher levels of gp78. To evaluate gp78's cell surface expression, we co-expressed it with HA-tagged caveolin-1 as a marker for its cell surface localization, as gp78 has been proposed to reside in the plasma membrane caveolae and endocytose in caveolin-1-dependent manner. We failed to observe any co-localization between these two proteins. However, consistent with the idea that gp78 is an ER resident E3 ligase; we observed extensive colocalization of gp78 with the ER resident proteins calreticulin, Sec61-β and Derlin-1 (data not shown for the last two proteins). Results obtained by a biochemical approach using cell-surface biotinylation also indicated that this protein was not present at the cell-surface. Although it should be pointed out that since we do not have the complete information on gp78's topology, it is difficult to assess the accessibility of Lys residues for the biotinylation reaction.

To rule out the possibility that overexpression and tagging of gp78 may have caused an artifact in our analysis, we acquired the 3F3A antibody (from the Raz group) that has extensively been used to study endogenous gp78's localization. The Nabi group has shown that the 3F3A antibody recognizes only a "subset" of overexpressed gp78 in the ER [154]. This was attributed to the production of immature or "conformationally inactive" form of gp78. This antibody however failed to react in our immunofluoresence analysis of endogenous as well as overexpressed gp78 under a variety of conditions tested (data not shown). It is quite possible that the reactivity of the 3F3A antibody was lost during transportation. However, when analyzing lysates from HeLa cells by Western blotting we did observe a 3F3A reactive-band around 78kDa. We also observed higher molecular weight bands that were reactive with this antibody. It is difficult to assess if these bands are non-specific bands or oligomers of gp78. Interestingly, as in the case of overexpressed gp78, we again failed to observe the endogenous 78kDa band from the products of cell-surface biotinylation reaction.

In an attempt to characterize the topology of gp78, we performed trypsin protection assay. Our results showed that both the N- and C-termini of gp78 are susceptible to trypsin digestion. This data, when considered with the results from topology prediction programs, indicates that gp78 has 4-6 transmembrane domains with the N- and C- termini in the cytosol (Figure 5.5). Gp78 has been previously proposed to be a seven transmembrane-domain containing GPCR. GPCRs are characterized by the presence of an N-terminal domain that extends out into the extracellular space and binds effector proteins while the C-terminal tail resides in the cytosol and interacts with the G-proteins. Due to the lack of any concrete evidence

demonstrating the cell-surface localization or the association of gp78 with G-proteins, it is highly unlikely that gp78 is a GPCR.

As previously described, it has been suggested that gp78 is N-glycosylated and this modification is essential for its interaction with AMF. As mentioned in the results section, gp78 has just one N-glycosylation site, which presumably resides in its cytosolic domain. Glycosidase treatment of gp78 revealed that this protein does not have high-mannose type glycan chains. Although we haven't yet tested for the presence of complex-type N-linked structures by PNGase F treatment, data from the Weissman lab suggests that it is not the case (Tsai and Weissman, unpublished data, personal communication). Thus, it appears that gp78 is not N-glycosylated, as previously proposed.

In summary, we have demonstrated that gp78 is a non-N-glycosylated, ER resident membrane protein. Gp78 consists of 4-6 transmembrane segments and both, the N- and C-termini of this protein are in the cytosol.

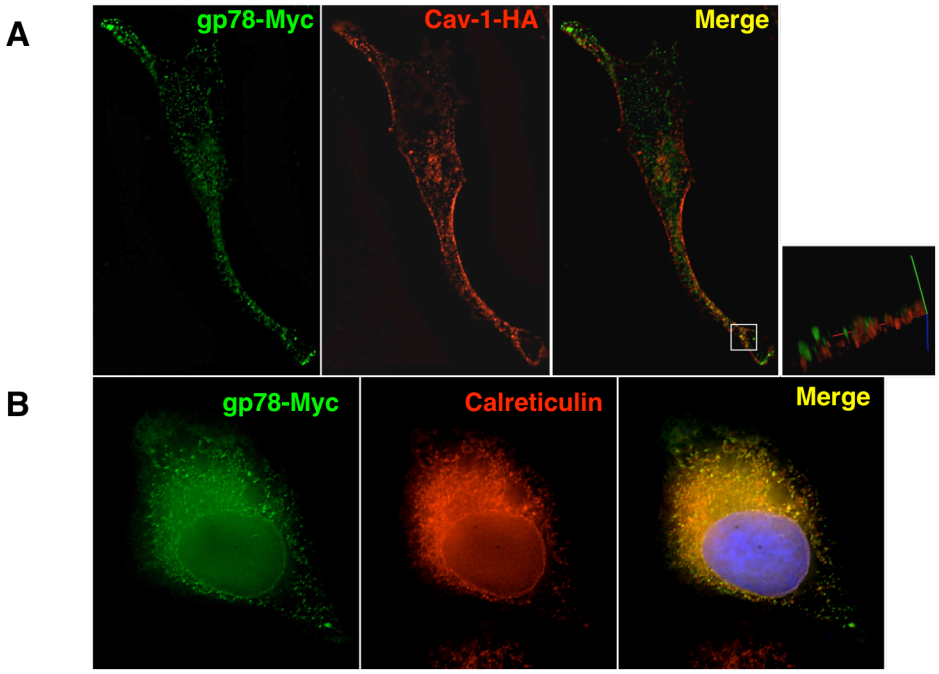

Figure 4.1 Gp78 Localizes to the ER. HeLa cells were co-transfected with caveolin-1-HA and gp78-Myc and stained with HA and Myc antibodies respectively. In panel A, the green channel shows the localization pattern of gp78, while the red channel shows the localization pattern of caveolin-1. The merge of both the channels (panel A) shows no co-localization of caveolin-1 and gp78. A magnified 3-D rendering of a small area of the cell cross-section (boxed area) from the merged image is also shown in panel A. Panel B, green channel again shows the staining pattern of gp78-Myc, while the red channel shows the localization pattern of the ER resident protein calreticulin. A merged image of the red and green channels shows a significant co-localization of gp78 and calreticulin.

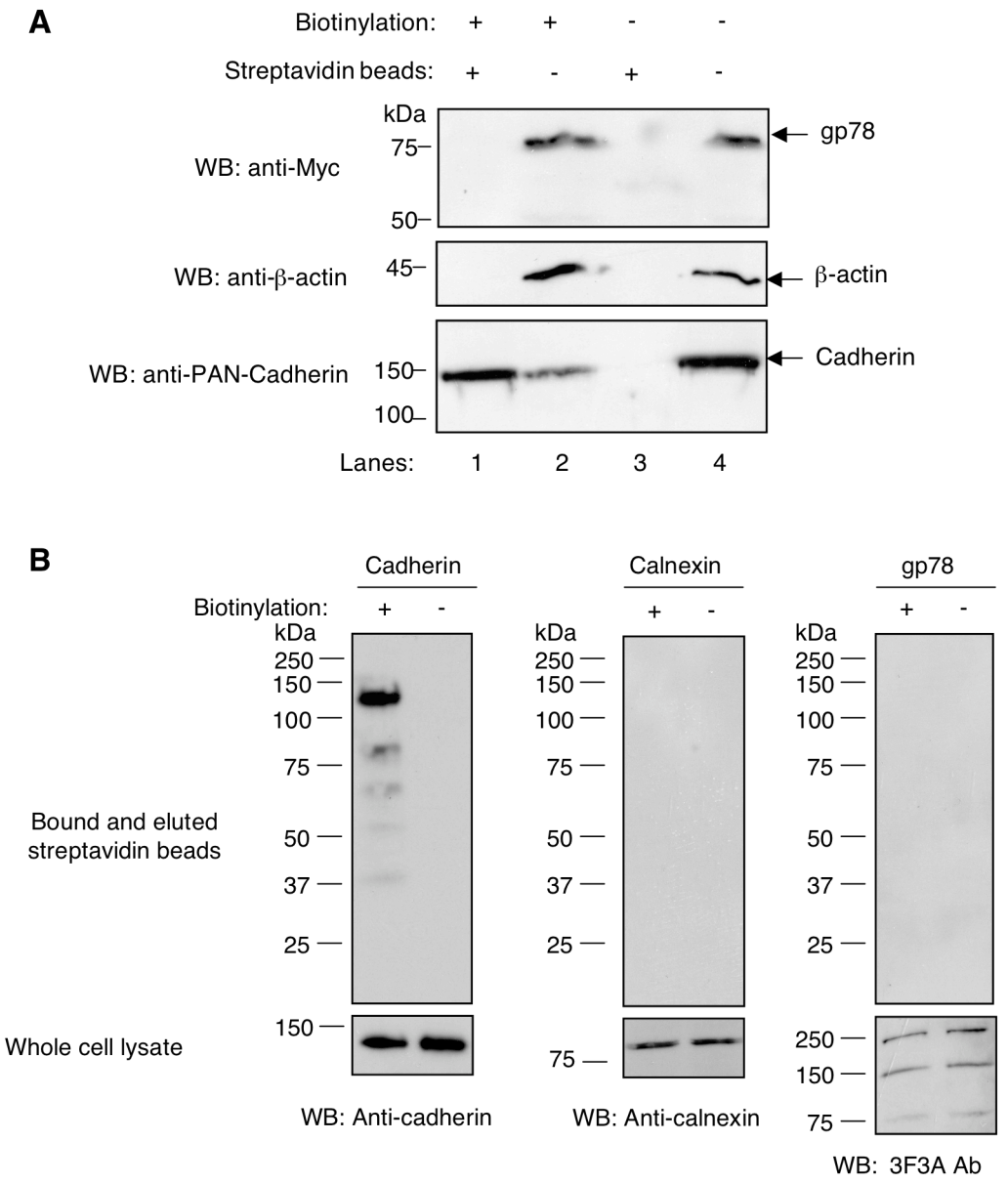

Figure 4.2 Gp 78 is not present at the cell surface. Cell surface biotinylation assay was performed on HeLa cells transfected with gp78-pcDNA4.0 (A) or left untransfected (B). In A, samples obtained after biotinylation (lanes 1 and 2) or mock biotinylation (lanes 3 and 4) were first processed with streptavidin beads (lanes1 and 3) or directly loaded on gel (lanes 2 and 4). After the Western transfer, the blot was cut appropriately and analyzed for gp78 (top panel), β-actin (middle panel) and cadherin (lower panel) using anti-Myc, anti-β-actin and anti-PAN cadherin antibodies respectively. Similarly, in B, samples obtained after the biotinylation (left lane, all panels) or mock biotinylation (right lane, all panels) were processed with streptavidin beads (top panels) or directly loaded on gel (bottom panels). Western blot analysis of all the samples was performed using antibodies to cadherin (left panels, top and bottom), calnexin antibody (middle panels, top and bottom) and the 3F3A antibody (right panels, top and bottom) respectively.

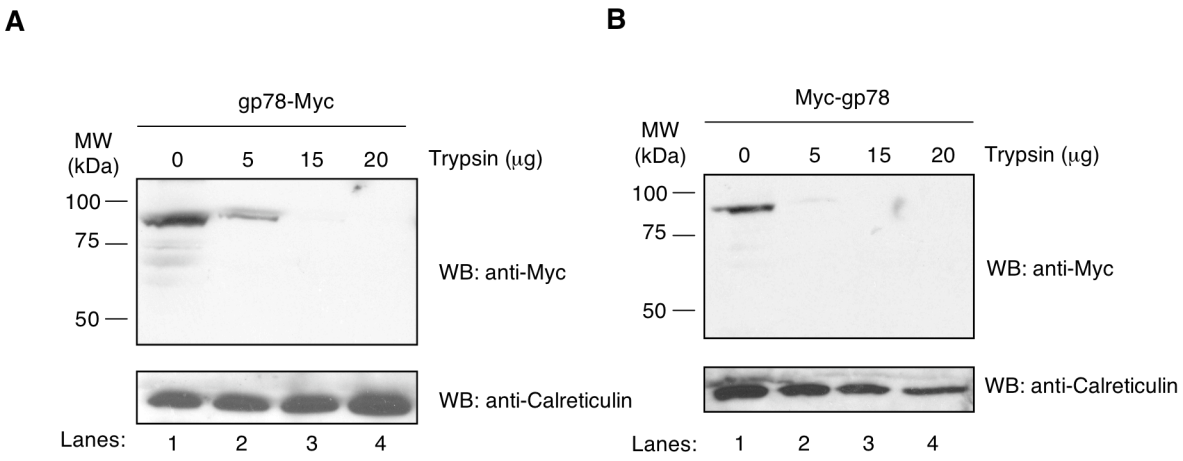

Figure 4.3 The N- and C-termini of gp78 are in the cytosol. HeLa cells were transfected with c-terminally Myc-tagged gp78 (A) or N-terminally Myc-tagged gp78. Following tissue homogenization, the post-nuclear supernatant carrying the intact ER membrane was treated with increasing amounts of trypsin (panels A and B lanes 1-4). The samples obtained from each reaction were loaded on SDS-PAGE and analyzed by Western blot analysis using anti-Myc antibody to detect gp78 (A and B upper panels). Immunoblotting with anti-calreticulin antibody (A and B lower panels) was also performed to rule out microsomal leakage during the homogenization procedure.

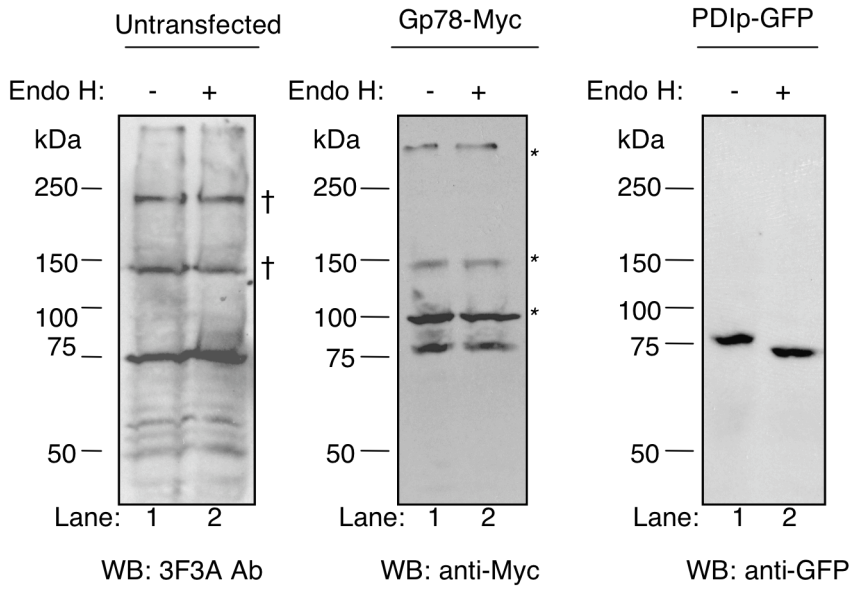

Figure 4.4. Gp78 lacks high-mannose type N-glycan chains: Lysates from HeLa cells expressing endogenous (left panel), and overexpressed gp78 (middle panel) were treated with EndoH (lane 2, left and middle panels respectively) or were untreated (lane 1, left and middle panels respectively). The samples were loaded on 8%SDS-PAGE and analyzed by Western blotting with anti-3F3A and anti-Myc antibodies and appropriate secondary antibodies for endogenous and overexpressed gp78 respectively. As a control, lysate from HeLa cells overexpressing a known N-linked glycoprotein of similar molecular weight, PDIp (GFP tagged, right panel) was treated with or without EndoH and analyzed by resolving the samples on 8% SDS-PAGE. This procedure was followed by Western blot analysis with anti-GFP antibody and appropriate secondary antibody. * Denotes the aggregation products of gp78 resulting from boiling the samples. † Refers to ubiquitinated products of endogenous gp78 or non-specific bands detected by the 3F3A antibody.

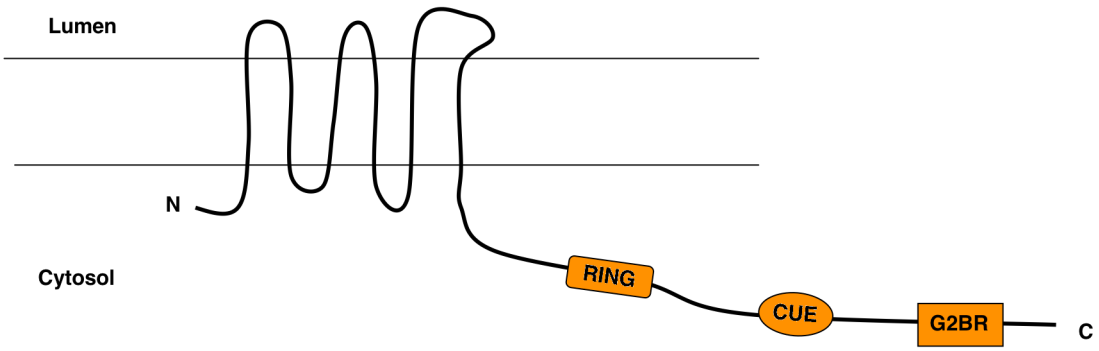

Figure 4.5. Predicted topology of gp78. Gp78 consists of 4-6 transmembrane spanning domains. The N- and C-termini are in the cytsol. The cytosolic tail consists of the RING finger domain that contributes to gp78's E3-ligase function, the CUE domain that binds ubiquitin and the G2BR domain, that is responsible for the interaction of gp78 with the E2 enzyme, Ube2g2.

Chapter 5. Analysis of the Derlin family of proteins in ERAD

Summary

The ER membrane protein Derlin-1 and its homologs Derlin-2 and Derlin-3 have been recently implicated in the dislocation process of misfolded glycoproteins. The overexpression of Derlin-1 has been shown to enhance the turnover of some ERAD substrates like HCs and CFTRΔF508, while having no affect on others such as TCR-α. Since Derlin-1 has been identified as a member of a degradation complex that consists of proteins involved in degradation of CD3-δ, we assessed the role of Derlin-1 in CD3-δ turnover. Our data shows that CD3-δ interacts with Derlin-1 and is degraded at a faster rate in the presence of overexpressed Derlin-1.

The mechanism of Derlin-mediated dislocation is poorly understood, although it has been speculated that these small, transmembrane spanning proteins form homo and heteroligomers that form a protein export channel in the ER. To identify conserved amino acid residues that may be crucial for this process, multiple sequence alignment of all the known Derlin orthologs was performed and 10 highly conserved amino acid residues were identified. Using site directed mutagenesis we have made a collection of Derlin-1 and Derlin-3 mutants targeting conserved residues in the membrane-spanning region and also the regions exposed to the cytosol and the ER lumen. Some of these mutants have been tested for defects in their ability to homo-oligomerize and localize to the ER. The mutations that have been have been examined thus far do not affect these properties of Derlin-1 and 3. Evaluation of the remaining mutants is important.

Materials and Methods

1. Plasmid constructs: Cloning of Derlin-1, Derlin-3 and CD3-δ has been previously described [114] [25] [82]. The Derlin-1 ORF obtained from the restriction endonulcease digestion (5'EcoR I, and 3'Xho1) of Derlin-1-pcDNA4.0 construct was cloned into pcDNA3.1 plasmid to generate untagged forms of Derlin-1. A STOP codon was added by site directed mutagenesis.

2. Site directed mutagenesis: Point mutants of Derlin-1 were created by PCR amplification of the Derlin-1-pcDNA3.1 and Derlin-1-pcDNA4.0 plasmids in the presence of appropriate mutagenic primers. Products of the PCR reaction were treated with Dpn1 endonuclease and transformed into *Escherichia coli* DH5α strains. Colonies obtained after transformation were used for extraction of plasmid DNA, which was sequenced to confirm the incorporation of the desired mutation.

3. Tissue culture and transfection protocols have been described in Chapter 4.

4. Antibodies and reagents: Anti-Derlin-1 (rabbit polyclonal) and anti-Myc (rabbit polyclonal) antibodies were purchased from Abcam (Cambridge, MA). Mouse monoclonal HA antibody was obtained from Roche. Calreticulin antibody (rabbit polyclonal) and Sec61-β antibody (rabbit polyclonal) were purchased from Affinity Bioreagents (Golden, CO), and Millipore (Temecula, CA) respectively. Anti-Myc

(mouse monoclonal), AlexaFluor-555 (AF-555) conjugated anti-mouse and anti-rabbit, and fluorescien isothiocyanate (FITC) conjugated anti-mouse and anti-rabbit antibodies were purchased from Molecular Probes (Invitrogen detection technologies, Eugene, OR). Complete™ protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). Cycloheximide was purchased from Sigma (St. Louis, MO) and digitonin was purchased from Calbiochem (San Diego, CA). Protein A and Protein G sepharose beads were purchased from Amersham Biosciences (Uppsla, Sweden) and Invitrogen (Carlsbad, CA) respectively.

5. Immunoprecipitation analysis procedure has been described in Chapter 3.

6. Confocal microscopy techniques have been described in Chapter 4.

7. Cycloheximide chase anlysis: HeLa cells were grown at 70-80% confluency and transfected with the appropriate plasmids. Forty-eight hours post-transfection, the cells were treated with 100μg/mL cycloheximide for 0, 2, 4, 6 and 8hrs respectively. Cell-lysate from each time point was analyzed by 10% SDS-PAGE and Western Blotting with appropriate antibodies.

Results

1. Derlin-1 interacts with CD3-δ and enhances its turnover. In the past, Derlin-1 has been shown to participate in the turnover of misfolded proteins such as CFTRΔF508 and HCs. Derlin-1 was also shown to be a part of a complex consisting of other ERAD proteins such as Hrd1, gp78, HERP, VIMP and p97 **[47]**. CD3-δ is a well-characterized ERAD substrate and has been shown to degrade by a pathway involving gp78, HERP and p97 [48,82,143]. To assess whether Derlin-1 is involved in the turnover of CD3-δ, we transfected HeLa cells with either HA-tagged CD3-δ alone or cotransfected HeLa cells with HA-CD3-δ and Derlin-1-GFP. Lysates obtained after 24 hrs of transfection were subjected to immunoprecipitation with anti-HA. Interactions between HA-CD3-δ and endogenous and overexpressed Derlin-1 (Derlin-1-GFP) were evaluated by performing SDS-PAGE and Western Blot analysis using anti-Derlin-1 and anti-GFP antibodies respectively. As seen in Fig. 5.1, HA-CD3-δ (left panel, lanes 1 and 2) interacts with endogenous Derlin-1 present in HeLa cells (middle panel) as well as the overexpressed GFP-tagged Derlin-1 (right panel). This interaction suggests that Derlin-1 may be involved in the turnover of HA-CD3-δ, by assisting its dislocation from the ER.

To test if Derlin-1 is involved in the turnover pathway of CD3-δ, we carried out cycloheximide chase analysis of HA-CD3-δ in the absence and presence of Derlin-1. For this purpose, HeLa cells were transfected with HA-CD3-δ alone (Figure 5.1B, left panel) or co-transfected with HA-CD3-δ and Derlin-1 (Figure 5.1B, right panel). Cycloheximide treatment was performed for 8hrs. As shown in Figure 5.1B there was an increased turnover of HA-CD3-δ in cells overexpressing Derlin-1 (Figure 5.1B right

panel) compared to the cells expressing endogenous levels of Derlin-1 (Figure 5.1B left panel).

Previous reports have indicated that N or C-terminal tagging of Derlin-1 with a large epitope such as GFP interferes with the Derlin function and causes it to behave like a dominant negative form [41]. Our results of the cycloheximide chase of HA-CD3-δ, carried out in the presence of C-terminally GFP or Myc-tagged Derlin-1 gave similar results. Upon coexpression of Derlin-1-GFP, we observed a substantial decrease in the turnover rate of HA-CD3-δ (data not shown). Taken together, our results indicate that Derlin-1 is involved in the turnover of HA-CD3-δ.

2. Multiple sequence alignment of the Derlin orthologs identified ten highly conserved residues: Sequences of all the known Derlin-1, 2 and 3 orthologs were retrieved from the NCBI protein database and multiple sequence alignment was performed. Figure 5.2A shows a representative sequence alignment of Derlin-1, 2 and 3 (transcript variants a and b) with the yeast ortholog (Der1p). Based on the alignment results, ten highly conserved amino acid residues have been identified that are identical amongst all known Derlin sequences. This is of significance, as the yeast homolog Der1p shares just ~13 % identity with the human Derlin-1, 2 or 3. Also, the identity between Derlins 1 and 2 or Derlins 1 and 3 is relatively low (~ 33%) while that between Derlins-2 and 3 is much higher, ~75%. These residues are distributed between the luminal, cytosolic and transmembrane spanning regions of the Derlins, as shown in Figure 5.2B. Thus, we rationalized that by making point mutations in these amino acid residues would provide clues to the function of the Derlin proteins. The impact of some

of these mutants on localization and homo-oligomerization and substrate turnover was studied and is described below.

3. Point mutants of Derlin-1 tested thus far localize to the ER. In order to rule out the possibility that a mutation in conserved residues of Derlin-1 would lead to impaired insertion in the ER membrane and possibly protein expression, we analyzed the localization of WT Myc-tagged Derlin-1 and the point mutants (R16Q, E87G, P38A, R51Q, L33K and L120K) in HeLa cells by confocal microscopy. Co-staining of the WT Derlin-1 and the point mutants with anti-Myc antibody and the antibodies for the endogenous the ER proteins Sec61-β or calreticulin was carried out. As in the case of WT (Figure 5.3, left panel) the Derlin-1 point mutants analyzed (Figure 5.3 and C left panel) were found to co-localize (right panels A, B and C) with the ER markers Sec61-β (Figure 5.3 middle panel) and calreticulin (Figure 5.3 middle panel) respectively. (Data not shown for the other mutants described above).

4. Derlin-1 and Derlin-3 form homo- and hetero-oligomers respectively. The Derlin family proteins constitute a class of very small membrane proteins of 25 kDa. Therefore, in a model for Derlin mediated retrotranslocation it is proposed that they can homo/hetero-oligomerize to form a protein-dislocating channel [41].

To assess if Derlin-1 can form self-oligomers or hetero-oligomers with Derlin-3, HeLa cells were co-transfected with GFP and Myc-tagged Derlin-1 or GFP tagged Derlin-1 and Myc-tagged Derlin-3. The lysate obtained from transfected cells was subjected to immunoprecipitation using anti-GFP antibody. Interactions amongst Derlin-1-GFP and Derlin-1-Myc and Derlin-1-GFP and Derlin-3-Myc were analyzed

by resolving the samples on SDS-PAGE and Western blotting with anti-GFP and anti-Myc antibodies respectively. As seen in Figure 5.4, lanes 1 and 2, Derlin-1-GFP (top panel) interacts with Derlin-1-Myc and Derlin-3-Myc (bottom panel) respectively. This demonstrates that Derlin-1 homo-oligomerizes and hetero-oligomerizes with Derlin-3. To study if Derlin homo-oligomerization was due to intermolecular disulfide bonds, lysates from HeLa cells expressing Derlin-1 and Derlin-3 were analyzed by non-reducing SDS-PAGE. We did not observe any high molecular weight bands indicating disulfide bond formation. These results established that homo-oligomerization was not disulfide-bond mediated (data not shown).

5. Point mutants of Derlin-1 homo-oligomerize. To test if the highly conserved residues are important for homo-oligomerization function we co-transfected Derlin-1-GFP with either the WT or a point mutant (P12A, R16Q, E87G) that was Myc-tagged. As described before, homo-oligomerization was assessed, using co-immunoprecipitation analysis. As seen in Figure 5.5, lanes 2-4, the mutations that I tested demonstrated homo-oligomerization capability, just like the WT Derlin-1 protein (Figure 5.5, lane1). This indicates that point mutations on the residues P12, R16 and E87 do not impact the homo-oligomerization property of Derlin-1. Mutations in the other conserved residues need to be tested in the future.

Discussion

Derlin-1, 2 and 3 have been collectively shown to participate in the turnover of several ERAD substrates such the mutant form of CFTR (ΔF508), HCs (disabled by the US11 and mK3 viral proteins) and the NHK variant of α-1 antitrypsin [25,40,45,155]. However, there seems to be a pattern of substrate specificity. For instance, the HC degradation is strictly Derlin-1-dependant, as the dominant negative form of Derlin-2 has no affect on its turnover rate [40]. Similarly, NHK turnover was shown to be Derlin-2 and 3-dependant, while Derlin-1 had no contribution in this process [25]. It is therefore likely that different Derlin proteins participate as components of ERAD complexes that are unique to a particular class of substrates.

Based on the lesions in their luminal, cytosolic and membrane domains respectively, misfolded glycoprotein substrates have been grouped into three main categories ERAD-L, ERAD-C and ERAD-M, respectively [74,156]. Comprehensive analysis of ERAD in yeast has indicated that substrates within each of these classes are dependant on a single degradation complex consisting of the same set of ERAD proteins. For instance degradation of ERAD-L substrate, CPY*, is dependent on a complex formed by ER lectins Yos9p, Htm1p (EDEM homolog), and other ERAD factors like Hrd1p, Hrd3p, Usa1p and Der1p. This also seems to be the case for mammalian cells, as Derlin-1 and 2 have been shown to be a part of an identical complex involving the mammalian orthologs of the aforementioned proteins (EDEM1, Hrd1, SEL1 and HERP). Also the substrate of choice for Derlin-2 and Derlin-3 is NHK, a luminal ERAD protein. Although the Derlin-1 substrate, HC is membrane associated, it has been classified as ERAD-L, as the US11 delivery of the HC (membrane protein) to

the Derlin-1 pathway was proposed to be reminiscent of Yos9p (ERAD-L component) targeting substrates to Der1p [157]. The mammalian ortholog of Yos9p is yet to be identified.

Degradation of ERAD-C substrates, for example, Ste6-166, requires completely different set of ERAD factors such as Doa10p and Ubx2p [157]. TEB4/MARCH-VI was recently reported to be a mammalian ortholog of Doa10p, however, substrates of TEB4 are yet to be characterized.

ERAD-M substrates demonstrate some amount of overlap with ERAD-L substrates, in the requirement for their turnover. Hmg2p, (ERAD-M substrate) degradation seems to be dependent on Hrd1p, a component of ERAD-L pathway **[74]**. However, other components of the ERAD-L pathway, Usa1p and Der1p have been thought to be dispensable for the ERAD-M substrates. In the mammalian system, the US2 protein appears to target HC's through the ERAD-M pathway that does not involve the Derlin proteins [36]. Similarly, another ERAD-M substrate, TCR-α, does not require the Derlins for its degradation [158].

Nevertheless, there appears to be some exceptions to this rule. CD3-δ, a well-characterized ERAD-M substrate was shown in a recent study to be targeted for degradation via HERP, a mammalian ortholog of Usa1p (previously thought to be dispensable for ERAD-M pathway) [48]. In the same study HERP was demonstrated to be a part of a complex consisting of Derlin-1, Hrd1, VIMP and p97. We therefore wondered if CD3-δ degradation also depended on Derlin-1. As shown in the results section, we observed an interaction between CD3-δ and Derlin-1, indicating that it may be targeted to a degradation complex consisting of Derlin-1. Sure enough, the overexpression of Derlin-1 caused an increase in CD3-δ's turnover rate. Also, the

expression of dominant negative form of Derlin-1 (Derlin-1-GFP) significantly delayed CD3-δ's turnover. Therefore, our results indicate that Derlin-1 is involved in the degradation of CD3-δ an ERAD-M substrate. Clearly several more ERAD substrates need to be characterized for their requirement of ERAD factors, before a pattern of substrate specificity can be generated. This task is harder for the mammalian system as compared to yeast, as significantly higher number of ERAD factors have been identified in the mammalian cells, some having redundant functions in the ERAD pathway.

Though several studies have demonstrated the significance of the Derlin orthologs in ERAD, it is noteworthy to mention that the mechanism of Derlin action is yet to be deciphered. The Derlins have been proposed to form a dislocation conduit, allowing misfolded proteins to be transported across the ER membrane for degradation [47]. Since the Derlins are relatively small, it is difficult to envision them as channel forming proteins. It has been suggested that the Derlin homo- and hetero-oligomerization properties facilitate the formation of a pore. A recent report has also indicated that ER stress, a condition that generates malfolded proteins, modulates Derlin oligomerization [159]. As discussed in the introduction, there have been suggestions that rather being a channel itself, the Derlins may associate with other pore forming proteins to carry out the process of dislocation. Good candidates for this are Sec61-β and α, which are the components of translocon machinery in the ER. We have explored the possibility of an association of Derlin-1 with Sec61-β and α, but have observed no interaction in the presence or the absence of a misfolded glycoprotein (CD3-δ). There are no other known interactions of the Derlins with other channel forming proteins.

To determine potentially important amino acid residues required for the function of Derlins in ERAD, we performed multiple sequence alignment of all the known Derlin orthologs from different species. The results of our alignment identified ten identical residues that were conserved amongst all the Derlin orthologs. As mentioned in the results section, this finding is important, as the yeast and mammalian Derlin orthologs share very little identity. We targeted these ten highly conserved residues for site directed mutagenesis, in the hope that a mutation in these would impact the localization and oligomerization properties of the Derlins and would also affect the ERAD of Derlin substrates. Thus far, we have only tested three mutations in the highly conserved residues of Derlin-1 for ER localization and oligomerization capabilities. These are P12A, R16Q and E87G. Similarly, mutations in partly conserved residues L31K, P36A, R51Q, and L121K have also been tested. Our data shows that these mutations (both in the highly conserved and similar residues) do not exhibit any defects in ER localization or homo-oligomerization. Also, the two mutants (P12A and R16Q) that were tested for their impact on CD3-δ's degradation, failed to show an effect (data not shown). Therefore, characterization of remaining mutants is necessary. It is noteworthy to mention here that Der1p in yeast was originally cloned as a gene complementing the ERAD defective mutant *der1-2* [42]. Sequencing of the *der1-2* allele indicated a mutation causing the substitution of S59L in the Der1p [160]. The S59 residue appears to be somewhat conserved, occupied by a similar residue, Thr, in other Derlin orthologs (T at position 57 of Derlin-1). Thus, for future analysis of Derlin-1, the T57 residue should be targeted for site directed mutagenesis study. As in the case of Derlin-1, it has also been noted that addition of a large epitope tag on the N- or C-terminus of Der1p results in the impairment of ERAD. This suggests two possibilities:

one, that the N- and C-termini of Derlin-1 are crucial for its activity and two, that the incorporation of a large tag sterically hinders the exit of proteins from the dislocation pore formed by the Derlins. We have previously shown that deletion of the cytosolic N-terminus of Derlin-1 causes its mislocalization in the cell. We believe that this region is essential for Derlin-1's incorporation into the ER. As observed in our previous study, the deletion of the C-terminus of Derlin-1, did not affect its incorporation into the ER. However, it inhibited Derlin-1's interaction with PNGase. It is therefore quite possible that the C-terminus of Derlin-1 is also crucial for interactions with other ERAD factors such as p97. Hence, it would be interesting to explore the effects of C-terminus deletion of Derlin-1 on ERAD.

In summary, our results thus far suggest that at least some of the point mutations (those tested) have no effect on the process of homo-oligomerization of Derlin-1. Neither do these mutations perturb the insertion of Derlin-1 in the ER membrane. To get a complete overview of this phenomena, the remaining mutants need to be tested for homo and hetero-oligomerization capabilities, insertion into the ER and effect on degradation of Derlin-1 substrates. With respect to the studies on the ERAD of misfolded proteins, we have identified a novel glycoprotein substrate of Derlin-1, namely CD3-δ. Identical mutational analysis of Derlin-2 and 3 also needs to be performed. The set of data obtained by these characterizations may eventually provide interesting similarities and differences between the three Derlin homologs, which may furnish much-needed answers to the questions related to channel formation and substrate specificity.

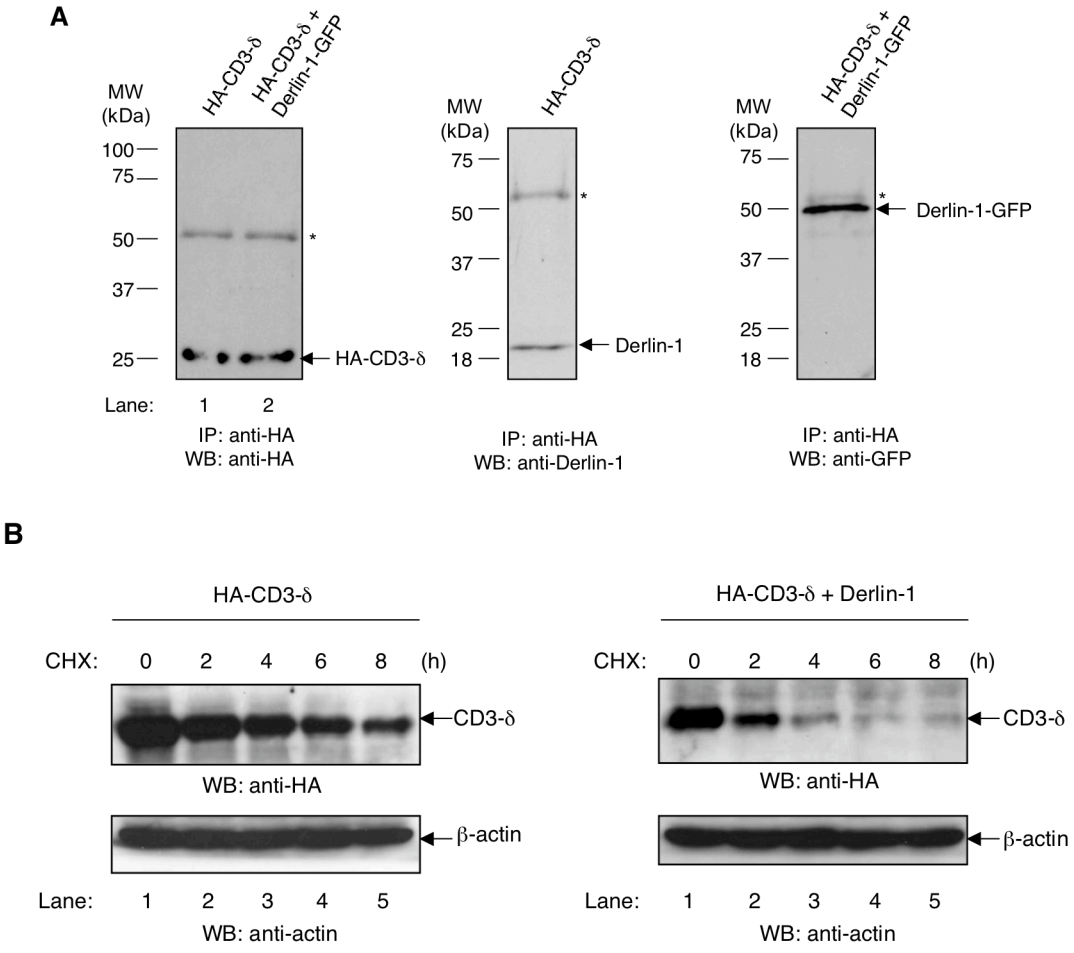

Figure 5.1 Derlin-1 interacts with CD3-δ and enhances its turnover rate.
A. Lysates from Hela cells transfected with HA-CD3-δ alone (left panel, lane1 and middle panel) or cotransfected with HA-CD3-δ and Derlin-1-GFP (left panel, lane2 and right panel) were subjected to immunoprecipitation analysis using anti-HA (mouse monoclonal) antibody. The samples obtained from the immunoprecipitation analysis were resolved on 10% SDS-PAGE followed by Western blot analysis using anti-HA (rabbit polyclonal, left panel), anti-Derlin (rabbit polyclonal, middle panel) or anti-GFP (rabbit polyclonal) (right panel) antibodies respectively. * Denotes non-specific band from secondary antibody. **B**. HeLa cells were transfected with HA tagged CD3-δ alone (left panel) or co-transfected with HA-CD3-δ and Derlin-1 (right panel). Forty-eight hours post-transfection, the cells were treated with 100 μg/mL of cycloheximide (CHX) for 8h. Cell-lysates from indicated time-points (0, 2, 4, 6 and 8 hrs) were resolved on 10% SDS PAGE and analyzed by Western blotting with anti-HA (top panels) or anti-actin antibodies (bottom panels A and B) respectively.

A

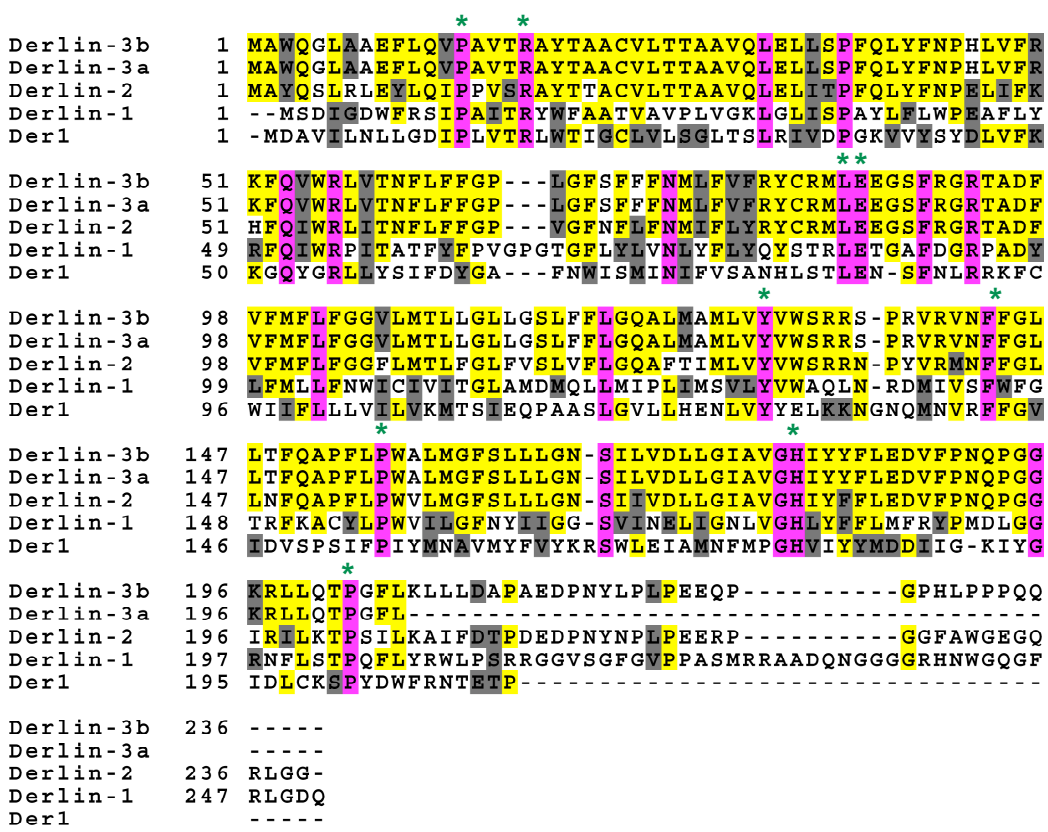

Figure 5.2A Multiple sequence alignment of human Derlins (Derlin-1, 2, 3a and 3b respectively), with the yeast ortholog, Der1p. Identical residues are highlighted with magenta, while similar residues are highlighted in yellow. The alignment was created using CLUSTALW tool from Biology Workbench (http://workbench.sdsc.edu). * Indicates the ten identical residues conserved amongst all known Derlin orthologs.

B

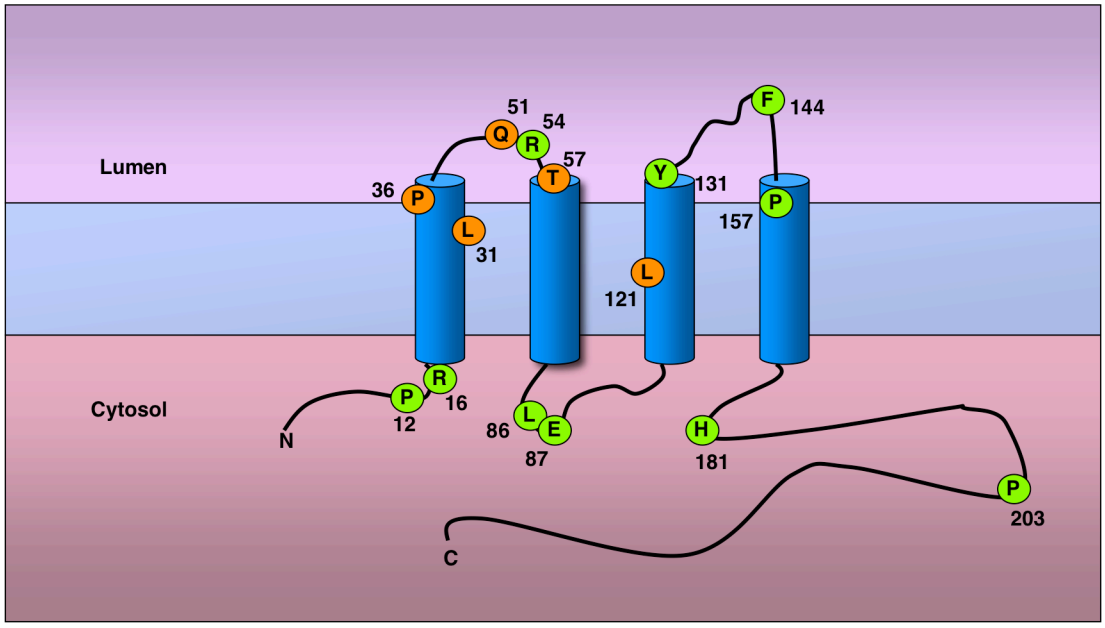

Figure 5.2B Predicted topology of Derlin-1. Also shown is the putative arrangement of all the identical residues (green) and some similar residues (orange) conserved amongst the Derlin orthologs.

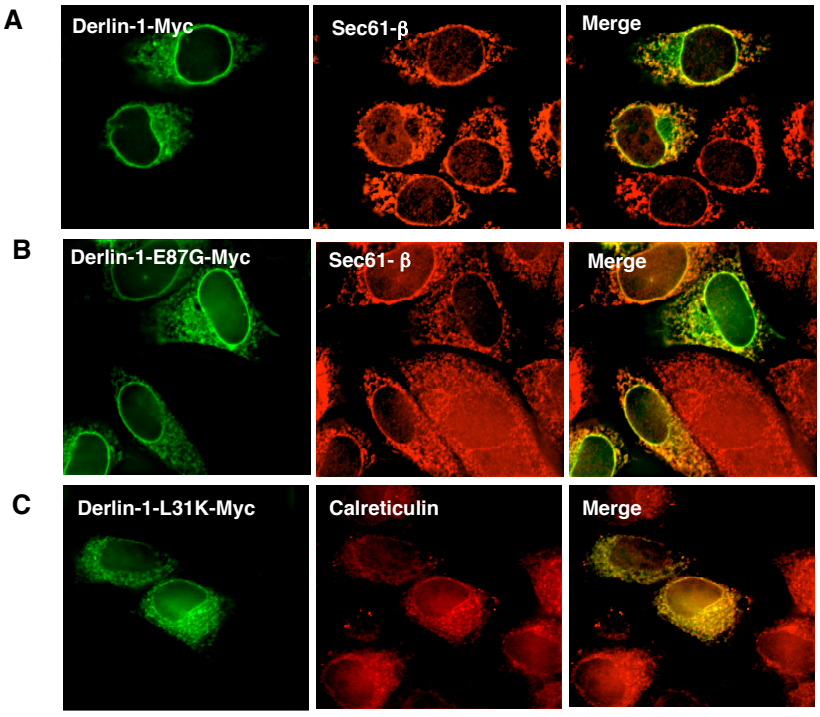

Figure 5.3. Wild type and point mutants of Derlin-1 localize to the ER. HeLa cells grown on coverslips were transfected with Derlin-1-Myc (wild type, left panel A), Derlin-1-E87G-Myc and Derlin-1-L33K-Myc (point mutants, left panel B and C respectively). The cells were fixed and co-stained with anti-Myc (left panels A, B and C) and anti-Sec61-β (middle panels A and B) or anti-calreticulin (middle panel, C) antibodies. The merge of left and middle panels (A, B and C) is shown in right panels (A, B and C) respectively.

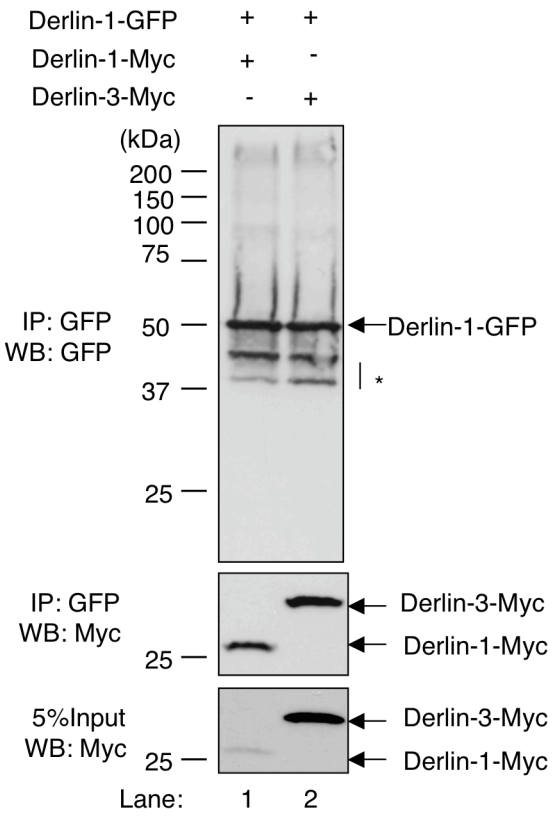

Figure 5.4 Derlin-1 forms homo-oligomers and hetero-oligomers with Derlin-3. Lysate from HeLa cells co-transfected with GFP-tagged Derlin-1 and Myc-tagged Derlin-1 or GFP-tagged Derlin-1 and Myc-tagged Derlin-3 (lanes1 and 2) respectively were subjected to immunoprecipitation analysis with monoclonal anti-GFP antibody. The samples obtained from immunoprecipitation analysis were resolved on 10% SDS-PAGE and analyzed by Western blotting using polyclonal anti-GFP (top panel) and anti-Myc (middle panel) antibodies respectively. 5% of the total input was also resolved on 10% SDS-PAGE. Western Blotting with polyclonal anti-myc antibody was performed to determine the expression levels of Myc-tagged Derlin-1 and Derlin-3- (bottom panel, lanes 1and 2) respectively. * Denotes partial degradation products of Derlin-1-GFP.

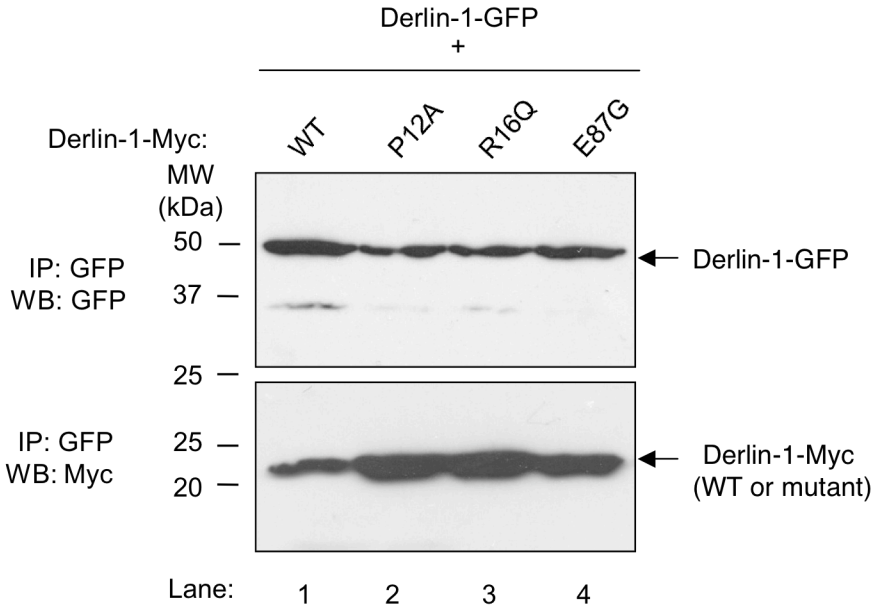

Figure 5.5 Wild-type Derlin-1 and its point-mutants homo-oligomerize. HeLa cells co-expressing Derlin-1-GFP and Derlin-1-Myc (WT) (lane 1) or Myc tagged point mutants of Derlin-1: P12A, R16Q and E87G (lanes 2, 3 and 4 respectively) were lysed in 1% digitonin buffer and subjected to immunoprecipitation with polyclonal anti-GFP antibody. Samples obtained from immunoprecipitation procedure were subjected to SDS-PAGE and immunoblotting with monoclonal anti-GFP (top panel) or anti-Myc (lower panel) antibodies respectively.

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